PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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MATING REACTIONS AND CONJUGATION BETWEEN VARIETIES OF PARAMECIUM AURELIA IN RELATION TO CONCEPTIONS OF MATING TYPE AND VARIETY[1]

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I. INTRODUCTION

THE conceptions of a mating type and a variety in *Paramecium aurelia* are based on the system of breeding relations. According to the published accounts (Sonneborn, 1938, 1942*a*), this system is clear cut and regular, and the current conceptions of a mating type and a variety follow naturally from it. However, there is on record (Sonneborn, 1938) one observation that raises questions as to the adequacy of the conceptions. The present paper reports many further such observations and attempts to formulate more satisfactory conceptions.

The published system of breeding relations may be summarized as follows. In certain races of *P. aurelia*, the clones are divisible into two classes. (As diverse clones may arise at the first fission of a fertilized individual, the clones here discussed are to be understood as originating, not with a fertilized individual, but with one of its vegetative descendants.) Clones belonging to the same class cannot conjugate with each other; but when two clones belonging to diverse classes are brought together under the proper conditions, the animals immediately agglutinate and soon pair off in conjugation. The classes of clones into which races are divisible in this way on the basis of their breeding relations are called "mating types." In some races, all the clones belong to the same mating type. Rarely, a single clone includes two mating types. All clones or individuals of *P. aurelia* are assignable to one or another mating type.

When tests are made as to the possibility of conjugation between the mating types found in different races, three kinds of results are obtained. (1) In many racial combinations, one of the two mating types in one race conjugates with one of the two mating types in the other race, and the other mating type in the first race conjugates with the other mating type of the second race. Such cases illustrate the occurrence of the same two mating types in diverse races.

[1] Contribution No. 320 from the Department of Zoölogy, Indiana University. The Rockefeller Foundation and Indiana University contributed to the support of this research.

(2) On the other hand, in most of the remaining racial combinations, neither mating type of the one race will conjugate with either mating type of the other race. Such cases illustrate the fact that the two mating types in one race differ from the two mating types in certain other races. Altogether, there are now known six diverse kinds of races, each with its own unique pair of mating types. Each of these sexually isolated groups of races is designated a "variety." Up to this point, there have been mentioned six varieties, each with two mating types, or a total of twelve mating types. (3) In the small proportion of races, mentioned above, that contain only one mating type, this type conjugates with one of the two types in certain other races and is therefore identical with the other mating type in those races.

There has been found only one race (among 108 examined) which fails to fit into the preceding account. Conjugation has been observed neither between different clones of this race nor, prior to the present study, between this race and any of the twelve known mating types. There is, however, as will be set forth below, convincing new evidence that this race represents a seventh variety and is pure for a thirteenth mating type, whose complementary mating type has not yet been found. More varieties and mating types of *P. aurelia* may yet be found. Present knowledge is summarized in Table 1.

A variety is thus conceived as a group of races that can conjugate with each other. If two races contain diverse mating types incapable of conjugating with each other, they belong to different varieties. Two clones are of the same mating type if they cannot conjugate with each other, although both can conjugate with one and the same third clone; two clones are of different mating types if one can conjugate with a clone with which the other clone cannot.

As already mentioned, there has been reported (Sonneborn, 1938) one observation which suggests that the system of breeding relations summarized in Table 1 is incomplete and, consequently, that the conceptions based on it may be inadequate. Mating type II of variety 1 was reported to give a weak mating reaction with mating type V of variety 3. The normal mating reaction between the two mating types of the same variety involves an immediate agglutination when clones of the two types are mixed together under the proper conditions: the animals clump together in clusters from which they later emerge as conjugated pairs, each of which consists of one animal of the one type and one of the other. When, under comparable conditions, clones of mating types II and V were mixed together, they went through the first part of this reaction, but not the second: they formed only temporarily adhering pairs and small clusters but soon separated without conjugating. (As will appear below, the present study adds some new features to our knowledge of the reaction between types II and V.) The capacity of mating types II and V to give this sort of weak mating reaction, even though not leading to conjugation, implies a similarity between the mating types of varieties 1 and 3. Type I of variety 1 and type V of variety 3 are similar in that both can react sexually (though to different degrees) with type II; and type II of variety 1 and type VI of variety 3 are similar in that both can react sexually (though again to different degrees) with type V.

This observation raises questions of

fundamental importance for an understanding of mating types and varieties. (1) Do other comparable intervarietal mating reactions occur? If so, they might lead to the recognition of similarities between other mating types comparable with those now indicated between the mating types of varieties 1 and 3; and such a system of similarities might well lead to a radical change in the conception of what the thirteen known mating types are. (2) Do intervarietal mating reactions ever lead to intervarietal conjugation? If so, this would bear not only on the conception of mating types but also on the present conception of varieties in this species, i.e., their complete sexual isolation. The present paper presents the data obtained in attempts to answer these two questions and discusses their bearing on current conceptions.

II. METHODS

The results that will be obtained from a study of intervarietal mating reactions depend upon the methods employed. In order to discover not merely the intensity but even the very existence of some of the intervarietal mating reactions, the investigator must provide the cultures with optimal conditions for mating; and many sources of error, which could lead to erroneous conclusions, must be guarded against by an elaborate series of controls.

1. METHOD OF CULTURE

The culture medium consisted of a lettuce infusion inoculated with the bacterium, *Aerobacter aerogenes*. The lettuce infusion is made from a powder prepared by washing the leaves from head lettuce, spreading them in a single layer on filter paper, heating them at

TABLE 1

THE SYSTEM OF BREEDING RELATIONS IN *P. aurelia*

The plus sign (+) indicates that the mating types in the corresponding row and file conjugate with each other; the minus sign (−), that they do not.

Variety		1		2		3		4		5		6		7
	Mating Type	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
1	I	−	+	−	−	−	−	−	−	−	−	−	−	−
	II	+	−	−	−	−	−	−	−	−	−	−	−	−
2	III	−	−	−	+	−	−	−	−	−	−	−	−	−
	IV	−	−	+	−	−	−	−	−	−	−	−	−	−
3	V	−	−	−	−	−	+	−	−	−	−	−	−	−
	VI	−	−	−	−	+	−	−	−	−	−	−	−	−
4	VII	−	−	−	−	−	−	−	+	−	−	−	−	−
	VIII	−	−	−	−	−	−	+	−	−	−	−	−	−
5	IX	−	−	−	−	−	−	−	−	−	+	−	−	−
	X	−	−	−	−	−	−	−	−	+	−	−	−	−
6	XI	−	−	−	−	−	−	−	−	−	−	−	+	−
	XII	−	−	−	−	−	−	−	−	−	−	+	−	−
7	XIII	−	−	−	−	−	−	−	−	−	−	−	−	−

about 180° C. in a hot-air sterilizer until most of the material is browned, removing the browned parts to a mortar by means of forceps, and grinding this material into a powder with a pestle. The infusion is prepared by adding 1.5 gm. of the lettuce powder per 1,000 cc. of double-distilled water (second distillation in Pyrex glass), boiling for 10–15 minutes, filtering through two layers of filter paper, adding a little $CaCO_3$ to the filtrate, and autoclaving for 20 minutes at 15 pounds pressure. After cooling, the infusion is filtered to remove the undissolved $CaCO_3$, dispensed into flasks, and autoclaved again. In this condition it may be kept until needed. To convert the stored infusion into culture medium, it is inoculated with *A. aerogenes* and incubated at 25°–30° C. for 18–48 hours before using. The bacteria to be used for inoculation are grown on yeast-agar slants at 25°–30° C. and are suitable for use when 2 or 3 days old. At this time they are subcultured to a new slant, and the remaining bacteria are washed off the slant with 5–10 cc. of sterile lettuce infusion. The bacteria are uniformly suspended in the wash fluid, and flasks of sterile lettuce infusion are inoculated with about 0.3 cc. of this suspension per 100 cc. of infusion. The suspension of bacteria washed from the slant may be used to inoculate more infusion on the next day, but not later.

2. METHODS INVOLVED IN PREPARING CULTURES FOR OBSERVATIONS ON MATING REACTIONS

Cultures to be tested for mating reactions were invariably derived initially from single individuals isolated in depression slides and grown in the dark in about 2 cc. of culture medium at 25°–30° C. until one to several thousand progeny were present. Then each culture was transferred to a plugged, sterile test tube; was kept under the same conditions; and, until the tube was about half-full, was given daily increasing amounts of food (just enough each day to supply the animals with a little more food than required to keep them well fed for one day). At this stage the cultures of most races may be brought into readiness for mating-reaction tests; but some races remain incapable of conjugating (immature) for a period after conjugation, and the duration of this period varies from race to race. It must be discovered and allowed for in the case of each race employed. During the period of immaturity, the tubes are fed daily and subcultured as required until the period of maturity is reached and there is on hand a tube half-full of culture.

To bring the tube cultures into readiness for maximal mating reactions, several further conditions must be supplied. One of these has already been indicated: the cultures must be grown in the dark from the start in order to avoid in certain varieties a diurnal periodicity in mating reactivity. Second, for at least 1 day before tests are made, the cultures must be grown at the temperature optimal for mating reactions. The optimal temperatures are 25°–30° C. for variety 1; 18°–20° C. for variety 2; 21°–24° C. for variety 3; 25°–30° C. for variety 4; and probably within the range 23°–28° C. for varieties 5, 6, and 7. (Varieties 5, 6, and 7 have not been studied so fully as the others, and the range of optimum temperatures is not yet precisely known for them; but they give, at the temperatures mentioned, reactions as strong as the maximum in the more fully investigated varieties. For an account of periodicities and temperature requirements in varieties 1, 2,

3, and 4, see Sonneborn, 1938, 1939; and Sonneborn and Dippell, 1943.) When tests for intervarietal mating reactions are to be made between varieties with different temperature optimums, cultures of both varieties are grown at both of the temperatures, and these are mixed together in all possible combinations.

The third condition that must be supplied is the proper nutritive condition. Animals do not give a mating reaction when there is still an abundance of food present, a condition indicated in the tubes by a cloudiness of the medium. On the other hand, they also fail to mate when they have been starved; and starvation also is likely to induce them to undergo autogamy, during which process the animals give no mating reaction. When the animals are starved, the tube cultures have lost their cloudiness, and the animals are either distributed through the tube or aggregated at the bottom. In order to get the animals in the condition of maximal sexual reactivity, therefore, the cultures must be worked with when the animals have nearly exhausted the food supply but before they starve. When the animals are in this condition, the tube cultures have lost their cloudiness, and the animals are aggregated just below the surface of the fluid; but the tube cultures first acquire this appearance shortly before the animals reach the state of maximal sexual reactivity. This state must be reached simultaneously by all the cultures that are to be tested with each other; and the achievement of such perfect timing requires that all the cultures to be used must be fed, on the previous day, an amount of food which will bring them into optimal reactive condition at the same time on the following day. In each case the amount of feeding depends on the population size, the temperature, and the growth rate characteristic of the race under the conditions being employed. Only by going though the experience of varying the amount of feeding and then testing at frequent intervals on the following day can one learn the correct amount of food to give in order to bring animals into maximal reactive condition at a specified time. No other technical phase of the work requires more experience and judgment.

Finally, a fourth condition must be supplied in one special case, namely, when working with race 40, the only available race of mating type XIII. Cultures of this race sometimes consist of large animals, sometimes of very small animals. When the animals are large, the cultures either fail to react or give only weak sex reactions; when they are small, they give maximal mating reactions. This difference in the size of the animals is due not merely to food supply, for cultures of large animals may be starved and the animals still remain larger than the animals in the small-animal cultures. Nor is the size difference correlated with sexual maturity, as it is in certain other races. For example, in race S of variety 1 the animals are relatively large even when starved during the 10 days or so of sexual immaturity following conjugation; but after they become mature they are large when well-fed and small when the food supply is giving out, and in the latter condition they are sexually reactive. On the other hand, conjugation never occurs within race 40, and so a period of immaturity in the usual sense is excluded. The cause of the small-animal condition in race 40 thus remains unknown. However, by carrying several cultures of this race at all times, it has been possible always to have some in the small-animal condition. This is greatly aided by the fact that when this condi-

tion develops in a culture it is likely to remain day after day for a long period.

When all the necessary conditions have been supplied and the cultures are in optimal reactive condition, mixture of two mating types of the same variety (regardless of which races of the variety are used to supply the mating types) results in the immediate agglutination of 85–95 per cent or more of the animals, and this is followed promptly by the formation of approximately the same proportion of conjugated pairs. (Here, as elsewhere in this paper, the given percentages of agglutination and conjugation are only approximate estimations. In dealing with large numbers of mixtures, each involving hundreds or thousands of animals, it is neither practicable nor valuable to make precise counts. As will appear, we shall be dealing only with large differences in percentages involved in reactions, and such large differences are readily apparent to the observer.) The only way to be sure that cultures are in optimal condition for mating reactions is to try them out and see if they give immediately 85–95 per cent reactions. As will appear below, tests for intervarietal mating reactions are adequately performed only when the cultures employed are known to be that reactive. Further, a negative result in an intervarietal combination of mating types may be considered reliable only when both highly reactive and less reactive cultures are repeatedly tried with the same negative result; for the experiments have given some evidence that the conditions optimal for intravarietal reactions may differ somewhat from the conditions optimal for intervarietal reactions.

The standard of maximal mating reactivity set forth in the preceding paragraph is inapplicable to mating type XIII because the presumed other mating type of its variety has not yet been found. The standard of optimal mating reactivity for this type must therefore be taken from the strongest mating reaction it can show with the available material. This, as will appear below, is its reaction with mating type II, with which it gives at best about 30 per cent immediate agglutination and about 10 per cent subsequent conjugation. When type XIII gave reactions of this intensity with type II, it was considered to be in optimal reactive condition.

The tests for mating reactivity were routinely set up in the following way. Material for the tests was obtained by withdrawing from the culture tubes the dense collections of animals near the surface. A test consisted of mixing together 0.15–0.20 cc. of the material from each of the two cultures to be tried. For unmixed controls, 0.3–0.4 cc. of the material from one culture was employed. Such mixtures and unmixed controls usually contained from 500 to 1,000 animals, the mixtures containing animals of the two cultures in approximately equal numbers. The mixtures and controls were placed in deep depression slides in water-sealed moist chambers in the dark at the appropriate temperature or temperatures for the varieties under investigation. When the mixtures involved two varieties with different temperature optimums, two sets were made and put at the two diverse optimum temperatures; when possible, a third set was placed at a temperature between the two optimums.

3. CONTROLS REQUIRED TO GUARD AGAINST SOURCES OF ERROR

In order to answer the question of whether two mating types belonging to different varieties can react sexually with each other, the obvious procedure is to mix together cultures of the two

types and observe whether a mating reaction and conjugation follow. The experiment will yield a valid answer, however, only if six diverse controls are carried simultaneously under the same conditions and if these six controls yield the results specified in the following paragraphs.

Controls 1 and 2.—The same two cultures used for the mixture must also be the source of unmixed controls that must be observed to ascertain whether conjugation occurs in either one alone; for, although cultures normally reproduce true to their mating type during the course of repeated fissions, a small proportion of cultures (selfers) include animals that can conjugate with each other (Kimball, 1939*b*). Moreover, in most races both mating types of the variety arise following the occurrence of autogamy within a clone, and autogamy may have occurred during the growth of the tube cultures used in the tests. If conjugation occurs in either of these controls, conjugation in the mixture may involve only one of the two cultures and so would be no evidence of intervarietal conjugation. Even if no conjugation occurs in the controls and does occur in the mixture, there remains the possibility (although, on the basis of much experience, this is improbable) that mixing the cultures has so altered the conditions as to permit the individuals from only one of the two cultures to conjugate with each other. Decisive evidence on this point can be provided by genetic studies. If the exconjugants are isolated and are found to inherit from both parental varieties, the possibility that they were derived from only one of the two original cultures is excluded. This genetic test was applied to all of the intervarietal conjugations that were found, but the genetic data are reserved for presentation in a later paper.

Controls 3 and 4.—To make certain that the two types being mixed are in condition to give maximal mating reactions, each of them must also be mixed with the corresponding mating type of its own variety. For example, when the possibility of interaction between type II of variety 1 and type V of variety 3 is under investigation, part of the culture of type II must be mixed with type I, the corresponding mating type of variety 1; and part of the culture of type V must be mixed with type VI, the corresponding type of variety 3. If these two mixtures yield 85–95 per cent immediate agglutination, followed promptly by an equal proportion of conjugation, then the cultures of types II and V are known to be in maximal reactive condition, and a failure to get a reaction between them would be significant; otherwise, not.

Controls 5 and 6.—The use, in controls 3 and 4, of two additional cultures (types I and VI in the example) involves the further necessity of establishing that these two cultures are pure for mating type and unable to conjugate without mixture. Therefore, a part of each of these two cultures must be observed without mixture. Otherwise, observation of conjugation in the control mixtures 3 and 4 would be subject to misinterpretation.

The full experiment, in the example used, would thus consist of the following parts:

Control 1.—Part of the culture of mating type II alone. This must not conjugate.

Control 2.—Part of the culture of type V alone. This must not conjugate.

Control 3.—Mixture of types II and I. This must yield an immediate agglutination of 85–95 per cent of the animals, followed by an equal amount of conjugation.

Control 4.—Mixture of types V and VI. This must give the same result as control 3.

Control 5.—Part of the culture of type I alone. This must not conjugate.

Control 6.—Part of the culture of type VI alone. This also must not conjugate.

Experimental mixture.—Part of the culture of type II mixed with part of the culture of type V.

The preceding model of the required experiment was followed in all cases except one, which required a modified procedure. Experiments involving variety 7 were limited, as mentioned above, by the fact that only one mating type (XIII) is known in this variety. Hence, two of the customary controls, those involving the as yet undiscovered type XIV, could not be set up—the mixture of type XIII with type XIV and the unmixed control of type XIV. These were replaced by mixtures of type XIII with type II, mixtures of type II with type I, and unmixed controls of types I and II. These four controls were required to give the usual results, except that the standard of maximal reactivity of type XIII was to yield 30 per cent agglutination with type II, followed by 10 per cent conjugation. This, as set forth above, is the strongest reaction ever observed with type XIII.

III. OBSERVATIONS ON INTERVARIETAL MATING REACTIONS

A. THE TWO GROUPS OF VARIETIES OF *P. aurelia*

Investigation of the results of mixing together, two at a time, cultures of the thirteen mating types in all possible combinations, using the standard methods set forth in Section II, showed that the seven varieties to which these types belong are divisible into two groups on the basis of their mating reactions: group A, consisting of varieties 1, 3, 5, and 7; and group B, consisting of varieties 2, 4, and 6. No one of the seven mating types of group A ever gave a sexual reaction with any one of the six mating types of group B. Further, no one of the six mating types of group B ever gave a mating reaction with any other mating type belonging to a different variety of group B. Negative results of this sort do not carry the same weight as positive results, for it is always possible that conditions may yet be discovered under which intervarietal mating reactions may occur between mating types never before known to react with each other. On the other hand, the same methods, which failed to show any intervarietal mating reactions in the combinations thus far mentioned yielded a number of positive results in combinations between mating types belonging to different varieties of group A. These results, which fall into a definite system, will be set forth in the next section.

B. MATING REACTIONS BETWEEN MATING TYPES IN VARIETIES OF GROUP A

The system into which fall the various sex reactions among the mating types of the group A varieties is shown in Table 2, which summarizes all the results to be set forth in the following paragraphs. It will be helpful to refer to this table as the reactions of each mating type are described.

1. *Reactions with mating type I (variety 1).*—Of the six possible combinations involving mating type I (I with II, V, VI, IX, X, and XIII), mating behavior was observed in only two: in the combination of type I with type II and in the combination of type I with type X. Types I and II are the two mating types of variety 1, and they give with each other the usual maximal intravarietal sex reaction under optimum conditions—90 per cent or more of the animals clump immediately after mixture and then proceed to conjugate.

On the other hand, types I and X be-

long to different varieties (varieties 1 and 5, respectively), yet they sometimes gave with each other mating reactions that led to conjugation. Genetic studies demonstrated that the conjugants in these mixtures were true intervarietal hybrids. As this is the first example of intervarietal conjugation to be cited in *P. aurelia* and as a number of other examples are to be given below, the general features of these reactions will be set forth here so that it will be unnecessary to repeat these features in connection with the later examples.

One general feature of intervarietal mating reactions is that their maximum intensity is always less than the maximum intensity of intravarietal reactions. The latter reach 95 per cent or more conjugation, while the former have never yielded more than 40 per cent conjugation. Only two intervarietal combinations, of which the combination of type I with type X is one, have yielded as much as 40 per cent conjugation, the others have always yielded much less.

The second general feature of intervarietal mating reactions is that what seem to be qualitative differences in the intervarietal reactions are correlated with quantitative differences in the intravarietal reactivity of the cultures. This may be illustrated by the behavior of types I and X. When type I is so weakly reactive that it gives only a small percentage of conjugants in mixtures with type II and when type X likewise gives a small percentage of conjugants in mixtures with type IX, mixtures of types I and X give neither conjugants nor any mating reaction whatever. When types I and X give a moderate percentage of conjugants in mixtures with moderately reactive cultures of the corresponding types of their own varieties, mixtures of type I with type X

TABLE 2

SUMMARY OF THE SYSTEM OF MATING REACTIONS IN THE VARIETIES OF GROUP A, *P. aurelia*

The numbers represent the maximum percentage of conjugant pairs formed in mixtures of mating types in the corresponding row and file. The symbol "Inc." represents an incomplete reaction that never leads to conjugation. The number accompanying the symbol Inc. gives the maximum percentage of animals involved in the incomplete reaction at any one time.

Variety		1		3		5		7
	Mating Type	I	II	V	VI	IX	X	XIII
1	I	0	95	0	0	0	40	0
	II	.....	0	1	0	40	0	10
3	V	.....	.....	0	95	0	0	0
	VI	.....	.....	.....	0	0	0	3-Inc.
5	IX	.....	.....	.....	.....	0	95	0
	X	.....	.....	.....	.....	.....	0	1-Inc.
7	XIII	.....	.....	.....	.....	.....	.....	0

still yield no conjugants, but a mating reaction occurs nevertheless. Animals of the two types adhere to each other when they accidentally meet during their random swimming, and small clumps may form; but the pairs and clumps remain united only for some seconds, or at most for a minute or two, after which the animals break apart with a characteristic jerk. In certain intervarietal combinations, this behavior may be repeated continuously over a long period, for 12 hours or more. Such a reaction may be designated as an "incompleted mating reaction." (Incompleted mating reactions have been reported in other connections by Sonneborn, 1937, 1938, and 1942*b*.) Many animals repeatedly undergo this incompleted reaction even in mixtures between cultures of types I and X that are in optimal reactive condition, as demonstrated by their maximal reaction with the corresponding types of their own varieties; but under these conditions many animals may also proceed to complete the reaction and conjugate. However, even under these optimal conditions, at most 40 per cent of the animals conjugate and usually not more than 15 per cent. Thus when intravarietal reactivity is weak, no intervarietal reaction takes place; when the former is moderately strong, only incompleted intervarietal reactions occur; but when intravarietal reactivity is maximal, the intervarietal reaction is incomplete for some animals and complete for others.

A third feature of intervarietal mating reactions is as yet only indicated. Sometimes intervarietal reactions were stronger in mixtures of cultures that gave submaximal reactions with the corresponding mating type of their own varieties than in mixtures of cultures that gave maximal reactions with their own varieties. This may mean that the optimum conditions for intervarietal reactions differ somewhat from the optimum conditions for intravarietal reactions of the same mating types. This possibility requires further investigation, as it may lead to the discovery of additional intervarietal reactions and to the raising of the maximum intensity level of some of those already known.

2. *Reactions with mating type II (variety 1)*.—The reaction of type II with type I has already been described. Of the remaining five possible combinations, two—the combinations of type II with types VI and X—give no mating reactions, but the other three do.

The reaction of type II with type V was the first intervarietal mating reaction to be discovered. It was reported (Sonneborn, 1938) to be of the incomplete type and to appear only when the cultures employed gave strong intravarietal reactions. The present study confirms and extends the earlier one. Mixtures of type II with type V give no mating reaction when the cultures mixed are only weakly reactive in intravarietal tests. When cultures are mixed that are yielding about 50 per cent conjugation in intravarietal tests, the intervarietal mixture shows the characteristic incomplete mating reaction: about 1 per cent of the animals adhere in pairs or threes at any one time, but these quickly break up and re-form continually for many hours without any conjugated pairs being formed. On the other hand, when the control intravarietal mixtures show maximal reactions, involving at once about 90 per cent of the animals, mixtures of types II and V give much stronger reactions. Up to 25 per cent of the animals may be agglutinated at any one time in clumps which may contain as many as eight

animals, but nearly all the clumps disintegrate and re-form continually. A few pairs, however, remain united and proceed to conjugate, but in no mixture do more than 1 per cent of the animals conjugate. The conjugants obtained in such mixtures have been shown by genetic studies to be true intervarietal hybrids.

The reaction of type II with type IX is the same as reported above for the reaction of type I with type X. Under optimum conditions as many as 40 per cent of the animals conjugate, though usually not more than 15 per cent do.

The reaction of type II with type XIII is intermediate in intensity between the two that have just been described. Under the best conditions, about 30 per cent of the animals agglutinate in clumps consisting of as many as 10 animals, but most of the reacting animals give only the incomplete reaction. In no mixture have more than 10 per cent of the animals proceeded to conjugate. Genetic tests have shown that the conjugants so formed are true intervarietal hybrids.

3. *Reactions with mating type V (variety 3).*—The results of mixing type V with types I and II have already been described. Of the four remaining possible combinations with type V, no mating reaction occurred in three of them: the combinations with types IX, X, and XIII. The fourth, the combination with type VI, gives the usual optimal intravarietal reaction, in which more than 90 per cent of the animals clump at once and proceed to conjugate.

4. *Reactions with mating type VI (variety 3).*—The results of mixing type VI with types I, II, and V have already been described. Of the other three possible combinations with type VI, no mating reaction occurred in two of them: the combinations with mating types IX and X. In the remaining combination, with type XIII, there occurred at best a weak, incomplete reaction involving the temporary pairing of not more than 3 per cent of the animals at any one time. Conjugation between types V and XIII has never been obtained.

5. *Reactions with mating type IX (variety 5).*—Type IX reacts only with types II and X. The former has already been described. The latter is the usual strong intravarietal reaction involving 90 per cent or more of the animals, which promptly proceed to conjugate.

6. *Reactions with mating type X (variety 5).*—The reactions of type X with types I and IX have already been described. Type X fails to react with any other mating type except type XIII, with which it gives the faintest of all sexual reactions thus far observed. This reaction is so weak that it occurs only when the cultures are in the most highly reactive condition, and it can be detected only by very experienced eyes. About 1 per cent of the animals may adhere in pairs at any one time, but the adhesions are so weak and brief that they result in only the faintest pull as the animals separate. Prolonged study is required to distinguish this reaction from the co-ordinated swimming-together of two animals which may occur in any culture independently of mating-type differences. Naturally, so weak a reaction has never yielded a single pair of conjugants.

7. *Reactions with mating type XIII (variety 7).*—All the reactions with this type have been set forth in the preceding paragraphs. Presumably this type would give the usual strong intravarietal reaction with type XIV, if this type were ever to be found.

8. *Summary of the mating reactions between mating types of the varieties of group A, with definitions of the mating types.*—

Table 2 summarizes the reactions described above. The numbers in the table are the maximum percentages of animals forming true conjugating pairs. The symbols 1-Inc. and 3-Inc. give the maximum percentages of incomplete pairs formed at any one time in mixtures that fail to conjugate. As appears in the table, the maximal intervarietal reactions that result in conjugation vary in intensity from less than one-half to nearly one one-hundredth the intensity of standard intravarietal reactions. On the very conservative estimate that a 1 per cent incomplete reaction is one-tenth as strong as a 1 per cent complete reaction, the weakest intervarietal reactions are but one four-hundredth as intense as the strongest intervarietal reactions and about one one-thousandth as strong as the standard intravarietal reactions.

Each mating type is defined by the mating types with which it will and will not conjugate. As examples, type I has previously been defined as the mating type which conjugates only with type II; type II as the mating type which conjugates only with type I; type III as the mating type which conjugates only with type IV; and so on. The relations set forth above show that these earlier definitions are incorrect. Some of the mating types can conjugate with two or more types, and satisfactory distinguishing definitions must include all or most of the relations summarized in Table 2: the types with which each type can conjugate, the types with which each type can give incomplete mating reactions, and the relative intensities of the various reactions under optimal conditions. As examples, a few complete definitions may be given:

Type I is the mating type which conjugates with types II and X, but more readily with II than with X.

Type II is the mating type which conjugates with types I, IX, XIII, and V, with the readiness of conjugation decreasing in the order named.

Type X is the mating type which conjugates with types I and IX, but more readily with IX than with I; and it can give only incomplete mating reactions with type XIII.

In like manner, each of the seven mating types of group A may be defined by means of the data in Table 2.

IV. COMPARISONS OF THE MATING TYPES OF GROUP A

The relations summarized in Table 2 are evidences of a system of similarities among the mating types of the varieties of group A. Mating types I, V, IX, and XIII are similar in that they all react sexually with mating type II. Mating types II, VI, and X are similar in that they all react sexually with mating type XIII. However, there is not a single instance in which two mating types are *identical* in their mating behavior. This is brought out by Table 3, which includes all the positive reactions listed in Table 2. Thus, the two types which are most nearly alike—types I and IX—differ in the intensities of their reactions with types II and X. All other pairs of similar types differ not only in their intensities of reaction with the same types but also in that one reacts with at least one type that the other cannot react with. For example, types II and X, which are more alike than any other types except I and IX, differ not only in their intensities of reaction with types I, IX, and XIII but also in that type II reacts with type V, while type X cannot react with type V. Hence, though the odd types are similar to each other, they are not identical; and likewise the similar even types are not identical.

This classification of the seven mating types into two groups of similar types has been made on the basis of the reactions of types II and XIII with the other types. For the classification to be consistent, it is required that no two similar types should react with each other and that all sexual reactions should occur only between dissimilar types. Tables 2 and 3 show that this is precisely what happens. All mating reactions are between an even and an odd type; no two odd types and no two even types react sexually with each other. The failure of three of the twelve odd-even combinations (I with VI, V with X, and VI with IX) to react is not inconsistent with the classification. The observations are thus all in agreement with the conclusion that the odd types form one similar set and the even types another.

According to this classification, the two mating types in any one variety of group A are similar to the two mating types in any other variety of this group, for the two types of each variety include one odd and one even type. Thus, in comparing varieties 1 and 3, type I of variety 1 is similar to type V of variety 3, and type II of variety 1 is similar to type VI of variety 3. The same similarities appear in comparing any two varieties of group A.

This system of similarities between different varieties of group A is based on their mating reactions. It is remarkable that types found to be similar in this way are also similar in four other ways. Some of these similarities had, in fact, been known before the system of sexual reactions was discovered in full; and it was because of this prior knowledge that, when designations were given to some of the mating types, the odd and even numbers were assigned on the basis of the suspected similarities. The four additional kinds of similarities are summarized briefly in the following paragraphs.

1. In races containing both mating types of a variety, the two mating types do not, as a rule, occur with equal frequency (Sonneborn, 1939). In such races of variety 1, there are commonly more, usually many more, clones of type II than of type I. Likewise, type VI is much more common in races of variety 3 than is type V. We now find that type X is by far the commoner type in variety 5. The rule is, thus, for the even type to be more abundant in races capable of yielding both types characteristic of a variety.

TABLE 3

POSITIVE SEXUAL REACTIONS AMONG THE MATING TYPES OF GROUP A

Note the differences between the reactions of the similar odd-numbered types and between the reactions of the similar even-numbered types. Symbols are the same as in Table 2.

Mating Type	VI	II	X
V.....	95	1	0
XIII.....	3-Inc.	10	1-Inc.
I.....	0	95	40
IX.....	0	40	95

2. Closely connected with this is the effect of temperature on the relative frequency with which the two mating types of a variety arise at the time of fertilization. It has been shown (Sonneborn, 1939) that the relative frequency of type II (in variety 1) and of type VI (in variety 3) increases with increase of temperature during a critical period at the time of fertilization or immediately thereafter. As type X of variety 5 is similar to types II and VI, it was suspected that its frequency would be cor-

related in the same way with temperature, and preliminary studies confirm this. In general, therefore, the relative proportion of the even types increases with increasing temperature at the time of fertilization.

3. Kimball (1939*a*) showed that when a clone of type II undergoes autogamy, the individuals destined to produce clones of type I fail to transform into type I immediately, remaining type II for several fissions. Sonneborn (1939) found the same so-called "cytoplasmic lag" in the transformation of type II clones into type I following conjugation, and also in the transformation of type VI clones into type V following either autogamy or conjugation. The reverse transformations, of type I into type II and of type V into type VI, proceed too rapidly for a cytoplasmic lag to be detected. Comparable studies have not yet been made on variety 5. In so far as the data are now available, it appears that cytoplasmic lag in change of mating type is readily detected only when the change is from an even to an odd type, not in the reverse direction.

4. In group A, races pure for one mating type are rare. Among the few that have been found, one belongs to variety 7 and is pure for type XIII, the others belong to variety 1 and are pure for mating type I. It is suggestive that all the races pure for mating type are pure for odd types; no race pure for an even type has yet been found.

The four preceding parallelisms among the mating types of different varieties agree with the similarities based on mating reactions. Altogether, they not only strengthen the conclusion that the mating types of different varieties are similar but suggest that fundamental similarities in their physiology and genetics may exist.

V. DISCUSSION

1. REVISION OF THE MEANING OF "VARIETY" IN *Paramecium*

As mentioned above, prior to the present study, the seven varieties of *P. aurelia* were thought to be set apart by complete sexual isolation. Sexual isolation was likewise the basis for distinguishing varieties of the other species of *Paramecium* that had been studied from this point of view. This conception of a variety in these organisms is no longer tenable. Jennings and Opitz (1944) reported that conjugation could occur between one mating type of variety IV and four mating types of variety II in *P. bursaria;* and we report in the present paper conjugation between variety 1 and varieties 3, 5, and 7 of *P. aurelia.* In view of these results, either the division of the species of *Paramecium* into varieties must be abandoned, or the meaning of the term "variety" must be modified. The latter seems the proper alternative because the only modification required is consistent with the intent of the original conception, which was simply to stress the impossibility for exchange of genes between varieties. The incapacity to interbreed is the most effective bar to exchange of genes; but if the latter is accomplished by other mechanisms, the diverse groups between which they operate are still effectively isolated and may be considered different varieties.

In *P. aurelia,* while the groups of races designated as "varieties" are not isolated sexually to the extent previously supposed, no two varieties are able to interbreed as freely as races belonging to the same variety. Difficulty in mating, usually very great difficulty, still operates as a partial bar to exchange of genes between varieties that can interbreed; and, so far as known, all but a few in-

tervarietal combinations are still completely isolated by this mechanism alone. In the few cases in which sexual isolation is not complete, a second mechanism for preventing exchange of genes appears—the hybrids themselves or subsequent inbred and back-crossed generations are of very low viability. Jennings and Opitz (1944) report that all the hybrids die in the only known intervarietal cross in *P. bursaria*, and they recognize this as a second isolating mechanism. In *P. aurelia*, the conditions among intervarietal hybrids and their progeny are more varied, as will be brought out in full in a later paper dealing with the genetics of intervarietal crosses. Here it may be said that in the cross of certain varieties most of the hybrids die, and the few survivors are so weak as to be unlikely to survive in nature in competition with either parental variety; while in the crosses between other varieties, the hybrids are vigorous, but the F2 and back-cross generations are mainly nonviable or weak. In the small proportion of vigorous survivors among the latter, there appears to be a third mechanism for preventing exchange of genes between varieties, for the survivors seem to be either like the hybrids or like the parental varieties.

It seems appropriate therefore to retain the present division of the species of *Paramecium* into varieties and to modify the conception of a variety from one based exclusively on complete sexual isolation to one based on potent bars of any kind to the free exchange of genes. These bars may be of several kinds, according to present information: complete sexual isolation; incomplete sexual incompatibility; hybrid non-viability or low viability; low viability of F2 or back-cross generations; and an incompletely understood mechanism by which the only survivors of the later generations are those that resemble the hybrids or the parental varieties.

Further study may disclose cases in which exchange of genes between varieties occurs rarely. This might be expected in early stages of varietal evolution. We have encountered one case in *P. aurelia* which may be interpreted in this way. Race 31 mates with the races of variety 4 as readily as any of these races mate with each other, but almost all the hybrids between race 31 and the other races are completely nonviable. (Mating within race 31, within the other races, and among the other races all give perfectly viable progeny.) There is thus an almost complete bar to exchange of genes between race 31 and the other races, without any detectable interference with free interbreeding. Although race 31 mates with no other races than those of variety 4, the question may be fairly raised as to whether it belongs to variety 4 or whether it should be considered as a member of a distinct variety. Possibly it represents an early stage in the evolution of a new variety.

2. THE MEANING OF THE BREEDING RELATIONS AMONG THE VARIETIES OF GROUP A IN RELATION TO MATING TYPES

The seven mating types in the varieties of group A were shown above to be divisible into two classes of similar mating types, the odd- and the even-numbered types. The most remarkable feature of these two classes of mating types is that they differ in the same way that the two mating types in any one variety differ: clones that belong to the same class cannot interact sexually or conjugate with each other; mating reactions and conjugation occur only between clones belonging to different classes of

mating types. This seems to mean that each class of mating types is itself a mating type of a more general sort. Therefore the even types (II, VI, and X) may be designated as "plus types" and the odd types (I, V, IX, and XIII) may be designated as "minus types." Parallels between the breeding relations of the plus and minus types and the breeding relations of the sexes in closely relates species of higher organisms are obvious. (There is no intention, however, of suggesting that plus and minus mating types are examples of male and female.) The nature and basis of the differences between different plus types and between different minus types will be taken up in later communications in connection with other studies.

The conception of plus and minus mating types cannot yet be extended to the varieties of group B because intervarietal mating reactions and conjugation have not yet been found in that group. There is, however, in the paper of Jennings and Opitz (1944) one observation suggesting that the mating types of *P. bursaria* may eventually be brought into a comparable system. They report that type R, one of the two mating types of variety IV, conjugates with four of the eight mating types of variety II (types E, K, L, and M). In agreement with the type of analysis here presented for *P. aurelia*, we are inclined to suggest that types E, K, L, and M of variety II are comparable with type S of variety IV because they all mate with type R; and that types F, G, H, and J, the other four types of variety II, are comparable with type R of variety IV because they all mate with types E, K, L, and M. This may be the beginning of the discovery of a system of similarities among the mating types of diverse varieties of *P. bursaria* that may lead to conceptions comparable to the conception of plus and minus types here proposed for *P. aurelia*. However, the existence of multiple mating types in certain varieties of *P. bursaria* complicates the whole picture and raises difficulties for the attempt, made above, to compare mating types of different varieties and for any attempt to extend to *P. bursaria* the conceptions based on the relations in *P. aurelia*. Further analysis of the complex relations in *P. bursaria* may be expected to throw much light upon the whole matter.

SUMMARY

1. The experimental methods required for satisfactory analysis of intervarietal mating reactions and conjugations are set forth in detail. These include method of culture; methods of bringing cultures into condition for optimal mating reactions (involving control of light, temperature, maturity, nutritive condition, nuclear condition, and size of animals); and the method of setting up an adequate series of controls to guard against misinterpretation of the observations.

2. The seven known varieties of *P. aurelia* are divisible into two groups on the basis of their sexual reactions: group A consists of varieties 1, 3, 5, and 7; group B consists of varieties 2, 4, and 6. No mating type of group A is known to react sexually or to conjugate with any mating type of group B; and no mating type of group B is known to react sexually or conjugate with any other mating type belonging to a different variety of group B. But a number of combinations of mating types belonging to different varieties of group A react sexually with each other; and several of these combinations also conjugate with each other.

3. The following sexual reactions have been observed between the varieties of

group A. Varieties 1 and 5 interbreed in both reciprocal combinations, type I with type X and type II with type IX; but only about 40 per cent as many conjugants form as in comparable mixtures between the two mating types of one variety, and usually much less. Varieties 1 and 7 interbreed in the combination of type II with type XIII, yielding 10 per cent as many conjugants as in intravarietal combinations. Varieties 1 and 3 interbreed only in the combination of type II with type V, yielding only 1 per cent as many conjugants as in intravarietal combinations. In addition, there occur two weak mating reactions that do not lead to conjugation, namely, between varieties 3 and 7 (type VI with type XIII) and between varieties 5 and 7 (type X with type XIII). No sex reactions occur between varieties 3 and 5.

4. Intervarietal mating reactions are characterized by three general features. First, the reactions are always less intense than between mating types of the same variety. Second, the reactions may differ in dependence upon the degree of reactivity of the cultures being used: when the cultures are weakly reactive with the corresponding mating type of their own variety, they may give no intervarietal reactions at all; when they are moderately reactive, they may give only incomplete intervarietal reactions that do not lead to conjugation; when they are at optimal reactivity, they may give complete conjugation in intervarietal combinations. Third, there is some indication that the conditions for optimal intervarietal reactions may differ somewhat from the conditions for optimal intravarietal reactions.

5. Each mating type may be defined by the types with which it can and cannot conjugate, by the types with which it can give incomplete mating reactions, and by the relative intensities of the various reactions under optimal conditions.

6. Types I, V, IX, and XIII are similar in that all react sexually with type II; types II, VI, and X are similar in that all react sexually with type XIII. However, similar types always differ in the intensity of their reactions with any type with which both can react, and, in all cases but one, one type can react with one or more types that the other cannot react with.

7. The system is such that sex reactions can occur only between an odd- and an even-numbered type. As each variety contains one odd and one even type, the odd one is similar to the odd one in every other variety of group A, and the even one is similar to the even one in every other variety of group A.

8. The following additional similarities between mating types of different varieties parallel those based on the mating reactions:

a) In races containing both mating types of a variety, the even-numbered type usually occurs more frequently than the odd one. Known for varieties 1, 3, and 5; untested in variety 7.

b) Further, in such races, increase of temperature during fertilization and immediately thereafter results in increasing proportions of clones of the even-numbered type. Known for varieties 1 and 3; indicated for variety 5; untested in variety 7.

c) When mating type changes at fertilization, there is a "cytoplasmic lag" in the transformation of an even into an odd type, but not in the reverse transformation. Known for varieties 1 and 3; untested in varieties 5 and 7.

d) Races pure for one mating type are,

in the only known cases, pure for the odd type of a variety, never for the even type. Known only in varieties 1 and 7.

9. The distinction of varieties on the basis of complete sexual isolation no longer holds, but the varieties may still be distinguished on the basis of potent bars to exchange of genes. These bars may be: complete sexual isolation; incomplete sexual incompatibility; hybrid nonviability or low viability; low viability of F2 or back-cross generations; and an incompletely understood mechanism by which the only survivors of the later generations are those that resemble the hybrids or the parental varieties.

10. One race (31) reacts sexually as if it were a member of variety 4, but there exists, nevertheless, between it and the other races of variety 4 an almost complete bar to exchange of genes; the effective bar in this case is hybrid nonviability. This case may represent an early stage in the differentiation of a variety.

11. The two classes into which the seven mating types of the group A varieties may be divided differ in the same way that the two mating types of any one variety differ, mating reactions and conjugation occurring only between the two classes, never between members of the same class. This is interpreted to mean that the two classes of mating types are themselves mating types of a more general sort, and they are therefore designated as "plus" and "minus" mating types. With respect to the breeding system, the plus and minus types of different varieties may be compared to the two sexes in closely related species of higher organisms.

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TROPHIC POTENTIALITIES OF THE GREEN FLAGELLATE LOBOMONAS PIRIFORMIS PRINGSHEIM[1]

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AMONG the several groups of flagellates classified both as Protozoa and as Algae, one, as the order Phytomonadina, is regarded as containing some of the most primitive Protozoa and, as the order Volvocales, is considered one of the most primitive groups of the green Algae. Moreover, these flagellates are thought to lie near the base of the evolutionary series leading to the terrestrial green plants. From the zoölogist's point of view, the Phytomonadina may thus be considered among the most plantlike, morphologically and physiologically, and among the most primitive, phylogenetically, of all existing Protozoa. Consequently, it has seemed likely that adequate information concerning the nutritional requirements of the phytomonad flagellates would furnish a sound basis for the cultivation and study of more highly specialized types. Subsequent comparisons might afford clues to the loss of primitive trophic potentialities and the possible acquisition of new nutritional processes among higher forms and thus might throw some light on the role that physiological specialization has played in the evolution of Protozoa.

Approximately thirty species of Phytomonadina have now been established in pure culture. The first careful study of food requirements seems to have been that of Schramm (1914*a*, *b*), who reported utilization of inorganic nitrogen by *Chlamydomonas pisciformis* var. *minor*. This is apparently the first conclusive demonstration of photoautotrophic[2] nutrition in one of the Phytomonadina. The next observations were made on *Volvox minor* and *V. globator* (Uspenski and Uspenskaja, 1925), both species being grown as photoautotrophs. In these early investigations no attempt was made to grow the flagellates on organic nitrogen sources, nor were cultures incubated in darkness as a test for heterotrophic nutrition.

Chlamydomonas agloëformis and *Haematococcus pluvialis* (M. and A. Lwoff, 1929; A. Lwoff, 1932) have since been found capable of carrying on photoautotrophic, photomesotrophic, and photometatrophic nutrition, and also heteromesotrophic and heterometatrophic nutrition in darkness. Neither flagellate has been grown in darkness on inorganic nitrogen sources. Photoautotrophic, photometatrophic, and heterometatrophic nutrition have been reported for *Chlorogonium elongatum* and *C. euchlorum* (Loefer, 1934), but attempts to demonstrate heteroautotrophic nutrition

[1] This paper is based upon a dissertation submitted to the faculty of the Graduate School of New York University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The writer wishes to express his appreciation to Dr. R. P. Hall for his suggestion of the problem and for his encouragement during the course of the investigation.

[2] The terminology applied to the various methods of nutrition is that suggested by Hall (1939). The prefix, *photo-*, designates nutrition involving photosynthesis; *hetero-*, nutrition carried on in the absence of photosynthesis and requiring an organic source of carbon. Utilization of different nitrogen sources is designated as follows: *-autotrophic*, inorganic nitrogen; *-mesotrophic*, amino acids, amides, or amines; *-metatrophic*, proteins or the higher protein derivatives. The term "chemoautotrophic nutrition" implies the ability to grow without photosynthesis in an inorganic medium.

were unsuccessful. Pringsheim (1934, 1935*a*), however, reported cultivation of *C. euchlorum* in darkness as a heteroautotroph, but he later concluded (1935*b*) that the flagellates tended to die in winter unless "soil extract" was added to the medium. Such results fail to demonstrate that this flagellate is a facultative heteroautotroph. Subsequently, Pringsheim (1937*a*) stated that both *C. elongatum* and *C. euchlorum* were grown in darkness on inorganic nitrogen and acetate with added "glucose caramel." On the other hand, Lwoff and Dusi (1935), like Loefer, were unable to grow *C. euchlorum* as a heteroautotroph, and they concluded that amino nitrogen was the simplest type suitable for growth in darkness.

Investigations on chlorophyll-bearing Phytomonadina thus have established that photoautotrophic nutrition is possible for all species studied, although heavier growth of at least certain ones is obtained under conditions insuring photomesotrophic or photometatrophic nutrition. Growth in darkness is possible with suitable organic nitrogen and organic carbon sources (heteromesotrophic and heterometatrophic nutrition), but the ability of green phytomonads to carry on heteroautotrophic nutrition has remained doubtful.

Among the colorless Phytomonadina, in which heterometatrophic nutrition is known for all species now in pure culture, *Hyalogonium klebsii* (Pringsheim, 1937*a*, *c*) appears to grow only with peptones and acetate or succinate and is to be considered an obligate heterometatroph. The requirements of several colorless species, on the other hand, are satisfied by single amino acids in suitable basal media, although growth is usually more vigorous with added acetate. Detailed information is available for *Polytoma uvella* (Pringsheim, 1921), *P. caudatum* (Lwoff and Dusi, 1937*b* and citations), *P. ocellatum* (Lwoff and Dusi, 1937*c*), and *Polytomella caeca* (Pringsheim, 1935*a*, 1937*b;* Lwoff and Dusi, 1937*a*).

Perhaps the only firmly established cases of heteroautotrophic nutrition in Phytomonadina are represented by *Polytoma uvella* (Pringsheim, 1921; Lwoff and Dusi, 1938) and *P. obtusum* (Lwoff and Dusi, 1938), both of which have been grown in inorganic media supplemented with acetate. *Polytomella caeca* (Lwoff and Dusi, 1938) apparently requires thiamin or both the pyrimidine and thiazole components of this vitamin, while *Polytoma caudatum* and *P. ocellatum* seem to need thiazole as a supplement to simple media. Accordingly, the last three species cannot be included in the group of facultative heteroautotrophs, since an autotroph by definition is capable of synthesizing any needed vitamins from the constituents of a simple medium.

These investigations have led to the consideration of possible similarities and differences between the chlorophyll-bearing and the colorless phytomonads (Hall, 1939; Pringsheim, 1941). Additional data on the trophic nature of the green types—and, particularly, more nearly complete surveys for individual species—might throw some light on problems of phytomonad phylogeny. One widely accepted theory is that the green stocks are the most primitive and that the evolution of colorless forms has involved merely the disappearance of chlorophyll with consequent elimination of phototrophic nutrition. According to another viewpoint, " . . . . the existence of heteroautotrophic flagellates, which can utilize inorganic nitrogen sources without carrying on photosynthesis, suggests the possibility that primitive colorless flagellates may have appeared

before the origin of chlorophyll. . . . . This hypothesis would gain additional support from the demonstration of chemoautotrophic nutrition in flagellates, and the suggestive report of such a phenomenon in *Chilomonas paramecium* [Mast and Pace, 1933] is particularly interesting" (Hall, 1941). In contrast to these two theories, interpretation of the phytomonad situation in terms of Oparin's (1938) generalizations probably would imply that the first phytomonad flagellates were colorless forms, living entirely by the absorption and assimilation of organic substances dissolved in the surrounding aqueous medium. From such primitive heteromesotrophs, origin of the chlorophyll-bearing photoautotrophs occurred much later and involved not only the appearance of chlorophyll but also the development of new metabolic activities. Colorless chemoautotrophs, likewise characterized by the acquisition of new and highly specialized physiological processes, arose from the heteromesotrophic stocks at approximately the same time as the photoautotrophs.

This general question and the lack of any detailed information concerning the food requirements of *Lobomonas piriformis* furnished the immediate problem for the present investigation.

MATERIALS AND METHODS

Lobomonas piriformis is a simple chlorophyll-bearing phytomonad flagellate isolated in pure culture by Pringsheim (1930). This strain was received by Dr. R. P. Hall in 1937 and since that time has been maintained in organic media at New York University. In such cultures, motile flagellates are usually few in number, whereas reproductive stages, consisting of two to four but sometimes eight or more individuals in aggregates, are common.

The writer obtained the flagellates as a stock culture in peptone medium. After tests of several media, stocks have been maintained in casein-peptone (Difco Tryptone, 2.5 gm/liter) solution containing the salts of medium EFO, EFBO, or EFCO. For the various experimental series four basal media were used as designated below, and nitrogen sources were added as indicated in Tables 1 and 2 and in the text.

MEDIUM EAO

$MgSO_4 \cdot 7H_2O$	0.1 gm.
KH_2PO_4	0.5 gm.
NaCl	0.1 gm.
Distilled water	1.0 liter

MEDIUM EFO

$MgSO_4 \cdot 7H_2O$	0.2 gm.
KH_2PO_4	0.2 gm.
$CaCl_2 \cdot 2H_2O$	0.1 gm.
Distilled water	1.0 liter

MEDIUM EFBO

Same composition as EFO, except that $CaCl_2$ was reduced to 0.04 gm.

MEDIUM EFCO

$K_2HPO_4 \cdot 3H_2O$ (0.1 gm.) substituted for KH_2PO_4 in formula for EFBO.

To each of the four basal media, $FeCl_3 \cdot 6H_2O$ and $MnCl_2 \cdot 4H_2O$ were added from stock solutions to produce final concentrations of 0.0025 and 0.0001 gm/liter, respectively. After a few preliminary tests (Table 1), medium EFBO was substituted for EFO because there was less precipitation and greater pH stability during sterilization. Subsequently, medium EFCO proved to be superior to EFBO in freedom from precipitate and from necessity for preliminary pH adjustment.

Salts of reagent quality were used—Mallinckrodt products with the exception of NH_4Cl (Eimer and Amend). Sodium hydroxide (Merck, c.p.) was used for pH adjustment, and pH de-

terminations were made with a LaMotte roulette. Organic materials were obtained from the following sources: amino acids, asparagin, amines, guanidine, and Peptone-Roche from Hoffman-LaRoche; ser. In subsequent series, for all transfers approaching and passing a critical dilution of the material carried over from stock cultures, the basal media were prepared with water redistilled in Pyrex.

TABLE 1

GROWTH IN LIGHT

Series	Basal Media	N-Sources (Gm/Liter)	Initial pH	Inocula (Cc)	Weeks of Incubation	Densities (1,000/Cc)
1.....	EAO (1–2)*	NH_4NO_3, 0.5	6.8–7.0	0.1	3	.....
2.....	EFO (1–8)	NH_4NO_3, 1.0	6.5–6.9	0.1–0.5	2–3	31–36 (summer)
3.....	EAO (1)	KNO_3, 0.5	6.9	0.1	3	.....
4.....	EFO (1–8)	NH_4Cl, 0.5 $(NH_4)_2SO_4$, 0.2	6.8–7.1	0.1–1.0	2–5	8–15 (summer)
5.....	EFO (1–2)	KNO_3, 0.8 $Ca(NO_3)_2$, 0.2	7.1	0.1–0.5	2	.....
6.....	EFO (1–4)	NH_4NO_3, 1.0	6.5–6.8	0.5–1.0	2–6	.....
	EFBO (5–14)	NH_4NO_3, 1.0	6.6–7.1	0.5–1.0	2–3	8(Dec.)–25(Aug.)
	EFBO (15–19)	NH_4NO_3, 1.0	6.7–7.1	1.0	3–7	8(Feb.)–19(May)
	EFCO (20–26)	NH_4NO_3, 0.24	6.7–7.1	1.0	4–7	51(Oct.)
7.....	EFBO (1–10)	KNO_3, 1.0	7.1–7.3	0.5	2	3(Nov.)–16(Aug.)
	EFBO (11–16)	KNO_3, 1.0	7.1–7.3	1.0	3–7	7(Feb.)–20(May)
	EFCO (17–20)	KNO_3, 0.61	6.6–7.2	1.0	4–7	33(Oct.)
8.....	EFBO (1–15)	NH_4Cl, 0.6 $(NH_4)_2SO_4$, 0.4	6.6–7.1	0.5–1.0	2–4	3(Nov.)–18(Mar.)
	EFCO (16–19)	NH_4Cl, 0.42 $(NH_4)_2SO_4$, 0.24	6.6–7.2	1.0	4–7	22(Oct.)
12.....	EFBO (1–10)	Asparagin, 1.0	6.7–7.2	0.5	2	3(Oct.)–9(Aug.)
	EFBO (11–17)	Asparagin, 1.0	6.9	1.0	3–7	3(Jan.)–15(May)
	EFCO (18–21)	Asparagin, 0.8	6.8–7.2	1.0	4–7	22(Oct.)
13.....	EFBO (1–10)	Glycocoll, 1.0	7.1–7.4	0.5	2	2(Oct.)–13(Aug.)
	EFBO (11–17)	Glycocoll, 1.0	7.1	1.0	3–7	3(Jan.)–26(May)
	EFCO (18–21)	Glycocoll, 0.9	7.1–7.2	1.0	4–7	28(Oct.)
14.....	EFBO (1–10)	Acetamide, 1.0	7.1–7.2	0.5–1.0	1–2	3(Oct.)–10(Aug.)
15.....	EFBO (1–5)	Phenylalanine, 1.0	7.1–7.2	1.0	4	10–17 (winter)
16.....	EFBO (1–10)	Methylamine, 1.0	6.9–7.1	0.5–1.0	3–5	1–3
17.....	EFBO (1–10)	Ethylamine, 1.0	6.9–7.1	0.5–1.0	3–4	1.5–9
18.....	EFBO (1–10)	Allylamine, 1.0	6.7–6.8	0.5–1.0	3–4	1.5–6
22.....	EFBO (1–10)	Tryptone, 1.0	6.8–7.0	0.2	1	69–327
23.....	EFBO (1–6)	Protone, 1.0	6.8–6.9	0.2–1.0	1–4	1.5–21
24.....	EFBO (1–10)	Casein, 1.0	6.8	0.2–0.5	1	43–124
25.....	EFBO (1–10)	Gelatin, 1.0	6.8–7.1	0.2–0.5	1–2	27–93
38.....	EFBO (1–9)	None added	6.9–7.4	1.0	3–4	2.5(Feb.)–25(May)
	EFCO (10–19)	None added	6.9–7.1	1.0	3–7	7(Apr.)–41(June) 32(Oct.)

* Numbers in parentheses refer to serial transfers.

acetamide from Eastman; urea from Eimer and Amend; gelatin, "isoelectric" casein, Tryptone, and Protone from the Difco Laboratories. Sodium acetate (Mallinckrodt) and soluble starch (Difco) were used as supplementary carbon sources. In series 1–5 the experimental media were made up with distilled water from a blocked-tin condenser. In subsequent series, for all transfers approaching and passing a critical dilution of the material carried over from stock cultures, the basal media were prepared with water redistilled in Pyrex.

The inorganic media were made up in Pyrex glassware reserved for such media and, after any necessary adjustment of pH, were tubed in 10.0-cc. amounts with individual burettes for different media. In preparing organic media the basal salt solution was tubed in 9.0-cc. amounts after adjustment of pH, and then 1.0 cc. of the organic nitrogen

source, as a solution in the basal medium, was added to each tube. Initial pH levels for the various media are given in Tables 1 and 2 and in the text. Final pH was usually the same, or else showed a drop of 0.1–0.2 units. More extensive changes, which were few, are indicated in the text. Individual flasks and pipettes were reserved for each type of organic medium, and culture tubes for inorganic and organic media were kept separate at all times. Tubes and pipettes were plugged with bleached nonabsorbent cotton (Johnson and Johnson). All culture media were sterilized in the autoclave at 122° C. for 20 minutes.

In the earlier experiments (sers. 1–5) cultures were incubated at room temperature near an east window. In later series all cultures exposed to light were incubated in a north window behind a single thickness of tracing paper, which reduced the normal light intensity by approximately 40 per cent. Daylight and room temperature were selected in view of Dusi's (1937) report that certain species of *Euglena* failed to grow under constant illumination. As indicated by preliminary tests, screened north light supported populations as dense as those obtained in an east window. Furthermore, bleaching of cultures, which some-

TABLE 2

GROWTH IN DARKNESS

Series	Basal Media	N-Sources (Gm/Liter)	Acetate (Gm/Liter)	Initial pH	Inocula (Cc)	Weeks of Incubation	Densities (1,000/Cc)
26	EFBO (1–6)*	NH_4NO_3, 1.0	2.0	6.6–7.1	0.5–1.0	4	2–10
	EFBO (7–10)	NH_4NO_3, 1.0	1.0	6.8–7.1	1.0	4–5	5–18
	EFCO (11–17)	NH_4Cl, 0.21 $(NH_4)_2SO_4$, 0.14	0.5	6.8–7.2	1.0	7–8	30 (14)*
27	EFBO (1–22)	Glycocoll, 1.0	0.0	7.0–7.2	0.5	4	0.1–0.5
28	EFBO (1–6)	Glycocoll, 1.0	2.0	7.0–7.3	0.5	4	2–4
	EFBO (7–10)	Glycocoll, 1.0	1.0	7.1–7.3	1.0	4–5	4–19
	EFCO (11–17)	Glycocoll, 0.45	0.5	6.9–7.1	1.0	7–8	66 (14)
29	EFBO (1–2)	Asparagin, 1.0	2.0	6.7–7.0	0.5	4	4.5–10
	EFBO (3–5)	Asparagin, 1.0	1.0	7.0	1.0	4–5	7–30
	EFCO (6–12)	Asparagin, 0.4	0.5	6.8–7.2	1.0	7–8	81.5 (9)
30	EFBO (1–2)	Peptone-Roche, 1.0	0.0	5.9	0.5	4–5	1.2–8
31	EFBO (1–2)	Peptone-Roche, 1.0	2.0	6.3	0.5	4–5	0.5–2
32	EFBO (1–3)	Tryptone, 1.0	2.0	7.1	0.5	4–5	47–780
33	EFBO (1–3)	Tryptone, 1.0	0.0	7.0	0.5–1.0	4,5	1–14
34	EFBO (1–3)	Tryptone, 1.0 NH_4NO_3, 1.0	2.0	6.7–6.9	0.5	4–5	27–544
35	EFBO (1–3)	Tryptone, 1.0 NH_4NO_3, 1.0	0.0	6.6–6.8	0.5–1.0	4–5	0.5–13.5
36	EFBO (1–7)	Tryptone, 2.5	1.0	6.9–7.0	0.2–0.5	4–5	50–2,170
	EFCO (8–14)	Tryptone, 2.5	0.5	6.9–7.0	1.0	7–8	No counts
37	EFBO (1–5)	Tryptone, 2.5	0.0	6.9–7.0	0.5–1.0	4–5	30–67
	EFCO (6– 2)	Tryptone, 2.5	0.0	6.9–7.0	1.0	7–8	No counts
39	EFCO (1–5)	None added	0.0	6.8–6.9	1.0	7–8	4–17
40	EFCO (1–5)	None added	0.5	6.8–7.0	1.0	7–8	11–38
41	EFCO (1–5)	NH_4Cl, 0.21 $(NH_4)_2SO_4$, 0.14	0.0	6.4–6.9	1.0	7–8	2–12
42	EFCO (1–5)	NH_4Cl, 0.21 $(NH_4)_2SO_4$, 0.14	0.5	6.8–7.0	1.0	7–8	9–35
43	EFCO (1–5)	Glycocoll, 0.45	0.0	6.8–6.9	1.0	7–8	1.5–14
44	EFCO (1–5)	Glycocoll, 0.45	0.5	6.8–7.0	1.0	7–8	10–61
45	EFCO (1–5)	Asparagin, 0.4	0.0	6.6–7.0	1.0	7–8	5–13
46	EFCO (1–5)	Asparagin, 0.4	0.5	6.6–7.0	1.0	7–8	9–34

* Numbers in parentheses refer to serial transfers.

times occurred under the latter conditions, was almost eliminated in north light. Cultures maintained in darkness were also incubated at room temperature. Incubation periods are listed in Tables 1 and 2 or in the text.

The experimental procedure, that of serial transfers, has been described previously (Lwoff, 1932; Loefer, 1934; Hall, 1938; and others). This method gradually eliminates materials carried over from the stock medium, so that the organisms are eventually forced to depend upon the experimental medium. For a positive conclusion concerning autotrophic capability of a species, it has been proposed (Hall, 1939) that the concentration of organic materials in the medium should be reduced to 1.0×10^{-10} gm/cc or less.

Records were kept for initial and final densities of population (number of flagellates per cubic centimeter) in the different experimental series. In Tables 1 and 2, final densities are recorded for transfers subsequent to critical dilution of materials from the original stock cultures. In addition, residual cultures, retained from successive transfers and fixed simultaneously at the end of each series, have furnished additional information concerning the time of attainment and the duration of maximal density. Since growth of *L. piriformis* was influenced by seasonal variations, the range in density of population was often rather wide in series maintained for a number of months (Table 1). For series incubated in light through both winter and summer, the months in which extremes of population density occurred are also given. The October counts for several series, with EFCO as the basal medium, are for a single transfer in each instance—transfer 13 in series 38 and the final transfer in each of the remaining series. Populations listed for May and those for August were from concurrent transfers, so that data within each group may be compared quantitatively. The populations for May happen to be those for the last transfer in medium EFBO in the respective series. The counting method was that of Hall, Johnson, and Loefer (1935).

PHOTOAUTOTROPHIC NUTRITION

Tests for photoautotrophic nutrition (sers. 1–8) were carried out with various inorganic nitrogen sources added to different basal media (Table 1), all cultures being exposed to daylight.

Series 1–5, started from stock cultures in peptone medium, served as preliminary tests of certain inorganic media. The medium in series 1 had proved satisfactory for *Chlorogonium* (Loefer, 1934), but *L. piriformis* failed to grow in the first transfer, and the population decreased in the next. Since the flagellates were no longer green and were probably moribund, the series was discontinued. The medium tested in series 2 is satisfactory for *Euglena anabaena* (Hall, 1938), whereas one almost identical with that used in series 1 failed to support growth of this species (Hall and Schoenborn, 1938). Growth of the flagellates was maintained, but bacterial contamination was discovered in transfer 10. Since routine tests in transfers 1, 2, 4, 6, and 8 had proved negative, contamination occurred in either the ninth or the tenth transfer. The experimental data thus were valid through transfer 8 and indicated that the medium was satisfactory for growth. Yields in the contaminated tubes were of interest in that the small bacterial population, difficult to detect microscopically, stimulated growth of the flagellates to a density of 55,000 in two weeks, 160,000 in three, and 1,250,000 in seven, the last figure being comparable to those for peptone media and

more than twenty times the density subsequently observed in the most favorable inorganic medium.

In series 3 the population decreased slightly during the first transfer. Although the flagellates were still green, the results were in such contrast to series 2 that a second transfer was not attempted. In series 4, growth was not so heavy as in series 2, but the results indicated that the series could have been continued indefinitely with large inocula and long periods of incubation. In series 5, growth in the first two transfers was comparable to that in the parallel series 4. However, the formation of a heavy precipitate during sterilization of the medium and a drop in pH to 6.0 during incubation were considered undesirable features, and the series was discontinued.

Series 6–8 were planned to confirm the apparent photoautotrophic nature of *L. piriformis*. In series 6, started from a peptone stock culture and carried in ammonium nitrate media over a period of almost two years, the results may be considered significant beyond the seventh transfer. In transfers 1 and 2 the flagellates varied greatly in size and color, and many stages of disintegration were observed. In the third and later transfers, the flagellates remained green and healthy. These phenomena suggest the possible occurrence of selection, as previously noted in *Euglena* (Hall and Schoenborn, 1939).

In series 7, started from transfer 3 of series 6, the average density of population in potassium nitrate media was somewhat lower and the greatest observed density less than in the parent series. In series 8, run in parallel with series 7, the combination of two ammonium salts supported smaller populations in most transfers, but heavier populations in others, than the nitrates tested in series 6 and 7.

The results demonstrate that *L. piriformis* is a facultative photoautotroph in media containing nitrate or ammonium salts, singly or in combination. An interesting feature, however, is that populations of *L. piriformis* in inorganic media were strikingly smaller than those developed by certain other phytomonads. For example, *Chlorogonium euchlorum* (Hall and Schoenborn, 1938) reached a density of 150,000 after an incubation period of one week. Furthermore, the same trend was observed in several other species tested by the writer in the medium used for later transfers of series 6 (Table 1, medium EFCO): *Chlorogonium tetragamum*, a density of 120,000; *Chlamydomonas agloëformis*, approximately 1,400,000; *Chlamydomonas pseudococcum*, about 5,200,000. In view of the present lack of detailed information concerning food requirements, these results may indicate that none of the media tested was actually optimal for *L. piriformis*. On the other hand, it is possible that *L. piriformis* is not so well adapted to growth in inorganic media as are the other green types investigated.

PHOTOMESOTROPHIC NUTRITION

In the tests for photomesotrophic nutrition (sers. 9–21), certain amino acids, amides, and amines were used as nitrogen sources in inorganic basal media (Table 1), all cultures being exposed to daylight.

Preliminary series 9 and 10 were started from transfer 10 of series 2, subsequently found to be contaminated. Series 9, with asparagin (1.0 gm/liter in medium EFO), showed early growth of *L. piriformis* in transfer 1, but heavy bacterial growth developed after prolonged incubation; in transfer 2, contamination was evident after 5 days. Series 10 with added glycocoll (1.0

gm/liter in medium EFO) was carried for six transfers. Bacterial growth was evident after 6–7 weeks of incubation. Series 11, with asparagin (1.0 gm/liter in EFO), was started from transfer 2 of series 10; the tubes became turbid in 3–5 days. Although the results were otherwise of no value, the contaminated cultures were interesting in comparison with later results in bacteria-free media. For example, in series 10, the density exceeded 1,500,000 in three weeks, whereas the maximum in bacteria-free glycocoll medium (ser. 13) was about 28,000.

Asparagin and glycocoll were tested again in parallel series 12 and 13, started from transfer 2 of series 6. The results for transfers 8–21 may be considered significant. Series 14 in acetamide medium was started from transfer 1 of series 8, and transfers 6-10 were concurrent with transfers 6–10 of series 12 and 13. Populations in these transfers were approximately the same in the three media. However, the highest densities observed later with asparagin and glycocoll were about twice as great. In series 15, started from transfer 12 of series 13, phenylalanine as a substitute for glycocoll yielded heavier populations in concurrent transfers. Series 16–18, with amines as nitrogen sources, were started from transfer 1 of series 8; of the three, methylamine was least favorable to growth. Series 19–21, yielding negative results with glucosamine, urea, and guanidine (each, 1.0 gm/liter in EFBO), indicated that all three substances were quite toxic for *L. piriformis* under the experimental conditions.

These tests for photomesotrophic nutrition demonstrated that inorganic nitrogen compounds may be replaced more or less satisfactorily by glycocoll, phenylalanine, asparagin, or acetamide. Smaller populations were obtained with methyl-, ethyl-, and allylamine, but growth was maintained in serial transfers. These results might suggest that any one of these seven substances will support photomesotrophic nutrition of *L. piriformis* in an inorganic basal medium. As discussed below, however, interpretation of the data was complicated by the subsequent growth of *L. piriformis* in a medium containing only trace amounts of nitrogen (ser. 38, Table 1).

PHOTOMETATROPHIC NUTRITION

This type of nutrition, in which proteins or their higher cleavage products supply nitrogenous food, was depended upon for maintenance of stock cultures in light and hence was already known for *L. piriformis*. However, the nutritive values of two proteins and two commercial peptones were compared in series 22–25 (Table 1), started from transfer 3 of series 13. The peptones differed markedly in their suitability for growth. With Tryptone (ser. 22) excellent growth was obtained. Residual cultures showed densities of 950,000 after 2–4 weeks, with a sharp drop to 300,000 or lower about the fifth week of incubation. With Protone (ser. 23), on the other hand, growth was too slow to balance the dilution in successive transfers, and the series was discontinued after the density had dropped to 1,600 in transfer 6. However, prolonged incubation of residual cultures produced populations of approximately 75,000 in 10 weeks. Although Protone is less thoroughly hydrolyzed than Tryptone, this feature apparently was not entirely responsible for the difference in results, since both casein and gelatin were better foods than Protone.

In casein medium (ser. 24) the greatest recorded density (124,000), after 2 weeks of incubation, was well below that obtained with casein-peptone (Tryptone). However, this maximum was

maintained for at least 10 weeks. Populations developed in gelatin medium (ser. 25), after short incubation periods, were usually smaller than those in the casein medium. In residual cultures, however, populations increased steadily to a final density of about 430,000 after 18 weeks.

HETEROAUTOTROPHIC NUTRITION

In the tests for various methods of heterotrophic nutrition, photosynthesis was suppressed by incubating cultures in darkness and, as supplementary checks on the suitability of each medium for *L. piriformis*, control cultures for each series were incubated in light.

Series 26 (Table 2), in inorganic nitrogen and acetate, was started from transfer 4 of series 6 as a test for heteroautotrophic nutrition. Relatively low populations in the controls exposed to light, noted in transfers 2–6 and attributed tentatively to excess acetate, led to a reduction in quantity of acetate at transfer 7. The change was beneficial to growth in darkness, and a further decrease in acetate (transfers 11–17) led to still heavier populations—e.g., a density of approximately 30,000 in transfer 14.

In this series, as in series 6, the flagellates showed morphological changes in the early transfers. In transfer 1, most of the flagellates were small and brownish, and only a few large green division-stages were seen. In transfer 2, size again varied, but the proportion of green dividing forms was much greater than in transfer 1. In addition to the dividing forms, other flagellates, equally large, were apparently undergoing disintegration instead of division. In transfer 3 there were a number of thickly encapsulated forms with fragmented chloroplasts, but the great majority were large and green. From transfer 6 on, the flagellates appeared normally green and healthy. These changes, like those in series 6, may represent processes of selection.

The results obtained in this series demonstrated that *L. piriformis* can carry on heteroautotrophic nutrition in darkness. Confirmatory results were obtained in series 40 and 42 (Fig. 3), described later.

HETEROMESOTROPHIC NUTRITION

In tests for heteromesotrophic nutrition (sers. 27–29 and 43–46), glycocoll and asparagin were added separately to inorganic basal media, with or without supplementary acetate (Table 2), and cultures were incubated in darkness.

Series 27 (glycocoll) was started with series 28 from transfer 2 of series 13. Although growth occurred, the population in transfer 2 was too small for further dilution in serial transfers, and the series was discontinued in anticipation of a second trial with a heavier initial population. The medium was satisfactory for growth in light, as indicated by a density of 15,000 for the controls in transfer 2. Series 28 (glycocoll and acetate) was run in parallel with series 26. A pronounced increase in yield occurred after the reduction of acetate at transfer 7, and an additional increase after further reduction at transfer 11. Morphological changes, similar to those described for series 26, were noted, but the flagellates were normal again in the fourth transfer.

Series 29 (asparagin and acetate), started from transfer 12 of series 12, was carried through 12 transfers coincident with transfers 6–17 of series 28. The greatest density (approximately 81,500 in transfer 9) exceeded that obtained with glycocoll (ser. 28) or with inorganic nitrogen (ser. 26) in the same basal medium with equivalent amounts of acetate.

Series 43–46 were started from transfer 11 of series 38 and were run in parallel with series 39–42, described below. In order to equalize the salt content, NaCl (0.215 gm/liter) was added to media lacking sodium acetate. The results (Fig. 1) indicate that *L. piriformis* grew in darkness in glycocoll and asparagin media without an additional carbon source, although growth was increased when acetate was added. In media without acetate, the pH declined 0.2–0.7 unit during incubation; with acetate, increases of 0.2–0.4 accompanied the development of the higher population levels.

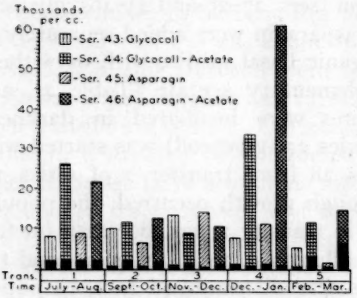

FIG. 1.—Heteromesotrophic nutrition (series 43–46). The base of each block (*solid black*) indicates approximately the initial density of population; the total height of each block, the density after incubation of each transfer. Transfer 1 was incubated during July and August, and the subsequent transfers during the months indicated for each.

HETEROMETATROPHIC NUTRITION

Two different peptones, and also sodium acetate and "soluble starch" as supplementary carbon compounds, were used in tests for heterometatrophic nutrition. Series 30 and 31 with Peptone-Roche (Table 2) were carried through only 2 transfers of 4 weeks each, because the population densities of less than 3,000 were smaller than those obtained with inorganic nitrogen. In distinct contrast to these results in darkness, the control cultures in light reached densities of 180,000–475,000. Tryptone, used in series 32–35 (Table 2) started from transfer 8 of series 22, yielded good results with added acetate (sers. 32 and 34). However, with added starch (0.1 per cent) in series 33 and 35, the media were unsatisfactory in that the populations were too small for continuation of serial transfers at the same rate of dilution in inoculation. The addition of ammonium nitrate (sers. 34 and 35) produced a 30–50 per cent decrease in density. It should be noted that starch, on the other hand, was not inhibitory to growth, either in light or in darkness. This substance merely seems to have no nutritive value for *L. piriformis* under these conditions.

In series 36 (Table 2), started from transfer 8 of series 22, the concentration of Tryptone was increased and that of acetate decreased. Populations were much more dense than in series 32. In these two series increases in pH of 0.5 (ser. 32) and 0.7–0.8 units (ser. 36) indicated at least partial removal of the acetate ion from solution. After good growth was noted in the first two transfers of series 36, a second attempt (ser. 37) was made to grow *L. piriformis* in Tryptone medium without added acetate. This series (Table 2), started from transfer 12 of series 22, was run in parallel with transfers 3–7 of series 36. The population yields indicated that Tryptone is an adequate source of food in heterometatrophic nutrition, although added acetate (ser. 36) is distinctly beneficial to growth. Although counts were not made, simple inspection of cultures showed that at least equally good growth continued in these series through seven additional transfers with EFCO as the basal medium.

GROWTH WITH TRACE AMOUNTS OF NITROGEN

Series 38 was started late in the present investigation in the hope of obtaining information which might lead to the improvement of inorganic media for *L. piriformis*. The results, which were altogether unexpected, have an important bearing on the interpretation of data previously obtained for inorganic and simple organic nitrogen sources. The unfavorable effects of ammonium nitrate, added to Tryptone media (sers. 34 and 35) in the usual concentration of 1.0 gm/liter, suggested determination of the optimal concentration of this salt for photoautotrophic nutrition. In series 38, started from transfer 11 of series 6, this was attempted by using only a basal medium (Table 1) and depending upon successive dilutions in inoculation to reduce the concentration of ammonium nitrate from the parent series. Unfortunately for the original purpose, the flagellates continued to grow (Fig. 2); and, with the increase in population yields during the spring and early summer, it became evident that *L. piriformis* could be maintained in a basal medium to which no specific nitrogen source was added. Computation of the nitrogen content from analyses of the component salts indicated a maximum of 1.8×10^{-8} gm/cc. The relation of these findings to other results obtained with *L. piriformis* is discussed below.

CHEMOAUTOTROPHIC NUTRITION

After the continued growth of *L. piriformis* with trace amounts of inorganic nitrogen (ser. 38), it seemed opportune to test cultures in darkness for chemoautotrophic nutrition, as previously reported in *Chilomonas paramecium* (Mast and Pace, 1933). Two series were planned—one with and one without ammonium salts added to basal medium EFCO. In addition, two companion series with added acetate were run as heteroautotrophic controls.

In series 39 (Table 2) the basal medium was supplemented with KCl and NaCl; series 40, with KCl and acetate; series 41, with ammonium salts and NaCl; series 42, with ammonium salts and acetate. Both NaCl and KCl were added in 0.215 gm/liter concentrations. All four series were started from transfer 11 of series 38. Changes in pH, occurring in the lightly buffered medium EFCO used in these series, may have had some bearing on population yields. In series 39, normal declines of 0.1–0.2 unit were recorded. In series 40 (with acetate) the pH level dropped 0.2 with a low population but rose as much as 0.4 unit with the higher population densities. In series 41, in which the densities were lowest, the pH level dropped 0.6–1.5 units. In series 42, even with acetate, pH depressions of 0.2–0.5 unit occurred. The fact that good yields with acetate were developed only at pH 7.0 or above suggests repetition of these series with better pH control. Only in this way will fully comparable data be obtained. Nevertheless, the results (Table 2, Fig. 3) in series 39 and 41 show that *L. piriformis* grew in darkness, under condi-

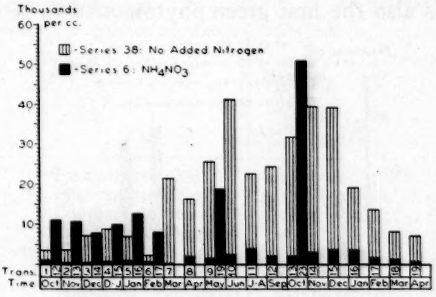

FIG. 2.—Photoautotrophic nutrition; corresponding transfers of series 6 with ammonium nitrate and series 38 in the basal medium alone.

tions suggesting chemoautotrophic nutrition, for five serial transfers over a period of 9 months, the density of population ranging from about 2,000 to 17,000 in the two media.

DISCUSSION

An unexpectedly wide range of trophic potentialities has been demonstrated in *L. piriformis*. The apparent occurrence of chemoautotrophic nutrition has been observed for the first time in the order Phytomonadina, and *L. piriformis* is also the first green phytomonad to be

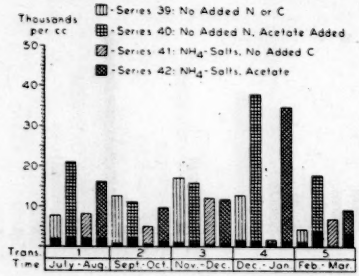

FIG. 3.—Chemoautotrophic nutrition (series 39 and 41) and heteroautotrophic nutrition (series 40 and 42).

grown in darkness in media comparable to those supporting heteroautotrophic nutrition of the colorless species, *Polytoma obtusum* and *P. uvella*. Such potentialities as these would ordinarily presuppose the widespread occurrence of a flagellate and thus raise the question as to why *L. piriformis* apparently is not, like certain of its relatives, worldwide in distribution. The answer, while not available, may reside in factors of competition. The comparatively low rates of increase in media without large amounts of organic nitrogen might result indirectly in an unfavorable competitive position. Direct antagonistic effects, contrary to the beneficial ones noted with one bacterial contaminant, are certainly possible. Such hypotheses might be tested with mixed cultures.

The ability to carry on photoautotrophic nutrition was expected, since all chlorophyll-bearing Phytomonadina so far tested are facultative photoautotrophs. The results obtained accidentally with bacterized cultures (ser. 2) have shown that a flagellate population in an inorganic medium may be affected favorably by a bacterial contaminant, perhaps through the contribution of synthesized growth-factors or other metabolic products; and it is obvious that conclusions concerning the photoautotrophic nature of flagellates can safely be based only upon data obtained with pure cultures. On the other hand, the writer is well aware that under natural conditions a flagellate is rarely, if ever, completely free from the influence of metabolic products of bacteria or other associated organisms and that just as seldom is the flagellate called upon to maintain a strictly autotrophic existence. Nevertheless, it is well to know to what extent this potentiality exists; conceivably, autotrophic nutrition might even be the "method of choice" for some species.

The continued growth of *L. piriformis* as a photoautotroph, after the omission of the usual nitrogen salt from the medium (ser. 38), seems to be without precedent in investigations on Protozoa. The calculated nitrogen content of this medium is about 1/80 that of New York City tap water (Loefer, 1936). In fact, the concentration of nitrate for fresh waters (Clarke, 1924) in general lies between that of the usual inorganic nitrogen medium and that of the "trace-nitrogen" medium (ser. 38) and thus well within the range for growth of *L. piriformis*. It is realized, of course, that *L. piriformis* might have utilized ammonia absorbed from the air of the laboratory or might conceivably have fixed atmospheric nitrogen. Unfortunately, preliminary ex-

periments, designed to prevent absorption of ammonia by culture media, have yielded inconclusive results, and additional data will be required to support any definite conclusion.

Whether *L. piriformis* utilized ammonia from the air or even fixed atmospheric nitrogen, the fact remains that this flagellate continued to grow in light in an inorganic basal medium unsupplemented with a nitrogen salt. These results necessitate careful consideration of certain other experimental findings for this species—for example, the comparative results with inorganic nitrogen sources (sers. 6–8). In Table 3, maximal yields with inorganic and the simpler organic nitrogen sources are compared with the maximal yield in the "trace-nitrogen" medium (ser. 38). With NH_4NO_3 the average density was greater, populations during the fall and winter months were distinctly more dense, and the highest observed yield exceeded that for the basal medium. In spite of the fact that seasonal variations in temperature and light invalidate detailed comparison of series not incubated concurrently, it seems safe to conclude that NH_4NO_3 was a fairly good supplementary nitrogen source, whereas KNO_3 and the mixture of NH_4Cl and $(NH_4)_2SO_4$ were without apparent nutritive value under the conditions indicated. This situation in *L. piriformis* may have a bearing on the interpretation of results reported for other flagellates, in that better growth with one inorganic nitrogen source than with another does not necessarily signify that either has accelerated growth. One or both might actually be inhibitory to a significant extent, and any such comparative data should be interpreted cautiously in the absence of information concerning growth in the unsupplemented basal medium.

TABLE 3*

RELATIVE POPULATION DENSITIES

Additions to Basal Medium	Incubation	Nutrition	Maximal Yield (In Per Cent)
None (ser. 38)	Light	Photoautotrophic	100
NH_4NO_3 (ser. 6)	Light	Photoautotrophic	124
Other inorganic N	Light	Photoautotrophic	36–87
Glycocoll	Light	"Photomesotrophic"	68
Asparagin	Light	"Photomesotrophic"	53
Acetamide	Light	"Photomesotrophic"	24
Amines	Light	"Photomesotrophic"	8–22
None (ser. 39)	Darkness	Chemoautotrophic	41
Inorganic N (ser. 41)	Darkness	Chemoautotrophic	30
Acetate (ser. 40)	Darkness	Heteroautotrophic	92
Inorganic N, acetate	Darkness	Heteroautotrophic	87
Glycocoll (ser. 43)	Darkness	Heteromesotrophic	34
Glycocoll, acetate	Darkness	Heteromesotrophic	160
Asparagin (ser. 45)	Darkness	Heteromesotrophic	31
Asparagin, acetate	Darkness	Heteromesotrophic	194

* Due allowance must be made for the fact that some of the comparisons are based upon transfers not incubated concurrently.

Interpretation of the data on photomesotrophic nutrition also is affected by the results obtained with trace amounts of nitrogen. Without the latter information, it would have been concluded that asparagin, acetamide, glycocoll, phenylalanine, and methyl-,

ethyl-, and allylamine were more or less satisfactory sources of nitrogen in photomesotrophic nutrition. Comparisons with the basal medium (ser. 38) show, instead, that none of these substances increased population yield in light (Table 3) and that the amines inhibited growth to a considerable extent. Although the flagellates were consistently larger in the asparagin and amino acid media and the total volume of flagellate protoplasm, as roughly calculated, somewhat greater than in the unsupplemented medium, these organic substances apparently were unimportant as foods under the specified conditions. Hence, it is impossible to conclude that photomesotrophic nutrition has been demonstrated in *L. piriformis*. However, this phase of flagellate nutrition requires further investigation with attention to the question of optimal concentrations of amino acids and comparable nitrogen sources.

As compared with the photoautotrophic method, photometatrophic nutrition is highly effective in *L. piriformis*, suitable proteins or protein derivatives increasing population yield by twenty to seventy-five times. The results obtained with gelatin and casein indicate that this flagellate is adequately equipped with proteolytic enzymes.

The artificial elimination of photosynthesis, by maintaining cultures in darkness, forced the flagellates to depend presumably upon metabolic activities which are normal to the colorless Phytomonadina. Under such conditions, *L. piriformis* apparently is able to carry on both heteroautotrophic and chemoautotrophic nutrition in appropriate media. The evidence for such phenomena in this species is similar to that from which comparable conclusions have been drawn for other microörganisms. The apparent occurrence of chemoautotrophic nutrition furnishes an index to the efficacy of photosynthesis in an inorganic medium and to the beneficial effect of supplementary acetate. A comparison of series 39 and 41 with comparable series (sers. 38 and 6) in light indicates that photosynthesis, as a supplementary source of energy, may more than double the yield of *L. piriformis* in such a medium (Table 3). The effect of supplementary acetate in darkness is of about the same order. In other words, sodium acetate in satisfactory concentrations is a fairly adequate substitute for photosynthesis when this flagellate is grown in an inorganic basal medium. Some of the present data for heteroautotrophic nutrition suggest that previous failures with green flagellates may have resulted from the use of acetate in excessive concentrations.

The growth of *L. piriformis* as an apparent chemoautotroph in a medium containing only trace amounts of nitrogen (ser. 39) questions the adequacy of glycocoll (ser. 43) and asparagin (ser. 45) as sources of both nitrogen and carbon in darkness, since growth with either of these supplements was no better than without. Similarly, various data (cited by Buchanan and Fulmer, 1930), which apparently indicate that certain bacteria utilize a number of amines and amides as sources of both carbon and nitrogen, may represent a situation of the same kind with supposed foods merely *permitting*, rather than supporting, growth of the test organism. With supplementary acetate, however, *L. piriformis* can carry on heteromesotrophic nutrition (Table 3). With glycocoll (ser. 28) the populations were not quite so heavy as with an equivalent concentration of asparagin (ser. 29), although distinctly larger than with inorganic nitrogen (Table 3). The apparently superior value of asparagin is in agreement with the

findings of Lwoff (1932). The occurrence of heteromesotrophic nutrition in darkness might prejudice the observer toward the assumption that photomesotrophic nutrition should be possible in light under favorable conditions.

The presence of chlorophyll apparently is no handicap to growth of *L. piriformis* in darkness as a heterometatroph, since populations in a suitable peptone-acetate medium reached densities comparable to those obtained without acetate in light and also to those reported for colorless members of the order.

A general survey of the present results indicates that *L. piriformis* is capable of carrying on any of the basic types of nutrition previously reported for Protozoa in pure cultures, with the possible exception of photomesotrophic nutrition. Furthermore, the writer's failure to obtain conclusive evidence for the occurrence of this type of nutrition may be the result of present ignorance concerning optimal concentrations of nitrogen sources rather than the inability of a green phytomonad to use simple organic nitrogen compounds in light. Hence, if this particular species can be considered representative, it would seem that the simpler chlorophyll-bearing Phytomonadina are physiologically poised for evolution in various directions and may justifiably be regarded as "primitive" flagellates.

Just how primitive the green types actually are remains, of course, a matter for speculation. Supporters of the theory that the ancestral flagellates were chlorophyll-bearing might argue that the various autotrophic abilities of *L. piriformis* lend support to the view that colorless plantlike flagellates have arisen from green stocks by regressive evolution. On the other hand, the apparent occurrence of chemoautotrophic nutrition in the chlorophyll-bearing *L. piriformis* (when grown in darkness) and in the colorless *Chilomonas paramecium* (Mast and Pace, 1933) affords some justification for the hypothesis that chemoautotrophic colorless flagellates were ancestral to the chlorophyll-bearing types. With the acceptance of such a premise, it would follow that evolution of the physiologically primitive green species, such as *L. piriformis*, involved nothing more than addition of photosynthesis to the pre-existing metabolic activities. Whether or not physiological specialization, comparable to that reported for Euglenidae (e.g., Hall, 1939), has occurred in the green phytomonad stocks is yet to be determined, since no species thus far tested has failed to grow as a photoautotroph. However, such changes, presumably involving the loss of certain basic abilities, have occurred among colorless Phytomonadina, leading to such species as *Polytomella caeca*, apparently no longer heteroautotrophic, and to even more exacting types (e.g., *Hyalogonium klebsii*) which have been grown only in complex organic media.

SUMMARY

Lobomonas piriformis, a green phytomonad flagellate, has been grown in light for nineteen serial transfers in an inorganic medium containing only trace amounts of nitrogen. As supplements to this medium, NH_4NO_3, peptones, casein, and gelatin increase the yield in varying degrees and apparently serve as nitrogenous foods. Certain amides, amines, and amino acids showed no nutritive value in light.

An inorganic basal medium, with or without added nitrogen salts, supported growth in darkness, indicating the occurrence of chemoautotrophic nutrition in *L. piriformis*. With added acetate (heteroautotrophic nutrition), growth in darkness approached that in inorganic

media exposed to light. Both glycocoll and asparagin increased the yield in darkness in the basal medium supplemented with acetate, while casein-peptone alone was about as effective as the combination of glycocoll and acetate. The combination of acetate and peptone produced yields comparable to those obtained in light with peptone media.

With the possible exception of photomesotrophic nutrition, not demonstrated by the writer's experimental data, *L. piriformis* has exhibited all trophic potentialities hitherto reported for flagellates.

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COMPARISONS OF MATING BEHAVIOR, GROWTH RATE, AND FACTORS INFLUENCING EGG-HATCHING IN SOUTH AMERICAN HAEMAGOGUS MOSQUITOES[1]

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THE following data were collected as part of a general program leading toward a fuller knowledge of the living characteristics of the insects which transmit jungle yellow fever.

Comparisons are made between some physiological properties of various species of the mosquito genus *Haemagogus* and related properties of the species *Aedes aegypti*. These characteristics are: (1) mating behavior and state of activity, (2) rate of development, and (3) egg-hatching characteristics.

Mosquitoes of the genus *Haemagogus* are implicated in the transmission of yellow-fever virus under the primitive forest conditions in tropical America in a way similar to the transmission of the same disease in urban areas by *A. aegypti*.

MATING BEHAVIOR

Seven species of *Haemagogus* and, in addition, *A. aegypti* have been observed under laboratory conditions. Notable differences in behavior, especially in mating activity, are present in these different forms. The species of *Haemagogus* observed were the following: *splendens*, *equinus*, *lucifer*, *boshelli*, *andinus*, *spegazzinii*, and *falco*, according to the terminology of Kumm *et al.* (manuscript), from whom most of the material was obtained.

The activities of the males and females up until the completion of the act of copulation may be divided into two types: the preliminary sexual tactics may include a kind of social behavior or "mating dance," or this may be replaced by a purely individual flight by the members of each sex.

Copulation may take place in flight and be completed there, or may take place in flight and be completed at rest, or may take place at rest after a preliminary flight and be completed at rest. The relative positions of the different species with respect to these methods of copulation are shown in Table 1.

The position of the male with respect to the female in copulation varies according to whether the pair is in flight or at rest. The resting pair remains in a ventral surface to ventral surface position; the pair in flight is in the position of male ventral surface over the dorsal surface of the female.

It was almost impossible to separate mating activity of the mosquitoes from general flight activity. Perhaps these activities are not much different in the male and virgin female mosquitoes. In the cages it was the species with the greatest general activity that showed the largest frequency of copulations and those with very little activity that could not be mated at all.

The flight activity and mating of the several species were tested in variously

[1] These studies were conducted as a Fellow of the National Research Council with laboratory facilities provided by the International Health Division of the Rockefeller Foundation and the Sección de Estudios especiales, Ministerio de Trabajo, Hygiene y Previsión social, República de Colombia, with the aid of Dr. Marston Bates, Dr. Henry Kumm, Dr. C. R. Anderson, and Dr. Ernesto Osorno-Mesa in providing material and suggestions.

sized cages, ranging from a small cage of 15 × 15 × 30 mm. to a room-sized cage for a species that showed little activity in the small cages. The results are tabulated in Table 1 in the order of activity for all species, with No. 1 showing the greatest activity and No. 7 showing the least. Except for Nos. 5, 6, and 7, the numbers also show the order of space requirement for mating. Space requirements for Nos. 1 and 2 have been judged by their behavior in the smallest cages, since *equinus* mated in the smallest cage used.

TABLE 1

THE COMPARATIVE MATING BEHAVIOR OF SEVERAL SPECIES OF *Aedes* AND *Haemagogus*

The numbers represent the degree of activity in the cages as well as space required for copulation.

Species	Copulation in Flight	Copulation Partly in Flight and at Rest	Copulation at Rest after Flight	1=Small Space; 7=Large Space
A. aegypti	Least	Common	Most frequent	1
H. splendens	Common	Common	Least	2
H. equinus	Common	Rarely	Never observed	3
H. lucifer	Common	Never observed	Never observed	4
H. boshelli	Common	Never observed	Never observed	4
H. andinus, *H. spegazzinii*, *H. falco*	Copulation has not been observed in these three			5, 6, 7

The mating behavior of *H. equinus* may be described as it took place in the smallest laboratory cages. The males would fly up and down the horizontal length of the cage whether light was applied to it from the side or from the top. The fanning dance was made in the cage facing the side at which the light was approaching unless it was from the top. Yet, in those cages having sides which were constructed of cellophane-covered screens the fanning behavior was most common against the sides at right angles to the source of the light.

In these cages mating was accomplished in the same manner as in the wild. A male would rush a female; and if the pair did not hit the side of the cage too forcefully, they might remain in copulation at flight or at rest. At times a leisurely flight was made in the attempt at copulation, after which the pair would fan in one position in the air for a time, the male being atop the female, with the abdomen curved around and under to meet that of the female. Actual union of the copulatory organs took place only in flight in this species.

The dance of *equinus* males in the crotch of an *Inga* tree on a coffee plantation near La Esperanza, Cundinamarca, Colombia, has been observed. Larvae of this species breed in tree holes of the *Inga*. A male would fly back and forth through the crotch, corresponding to the flight lengthwise in a cage, and going no farther than ±60 mm. in a horizontal direction. It would often stop and fan, in the manner of a hawk moth or a hummingbird. At the presence of a female a swift dart would be made, and both would disappear. In a short time the male (or another) would return, to continue the dance in the crotch of the same tree.

Copulation was never observed in *H. falco*, regardless of cage size. Both males and females seem inclined to inactivity

under any of the conditions which were provided for them, though occasional flights from one side of the cage to the other were made. Most of the activity occurred in a room-sized cage with artificial lighting. Trials were made in vials and in all intermediate-sized cages up to a burro trap placed in a jungle habitat. The greatest activity was observed in a bright, subdued light, such as that outside of buildings but with a light cloud over the sky. Full sunlight or full artificial light caused both *falco* males and females to retreat to dark corners.

In line with the inactivity of *falco* in cages is the lack of any evidence as to their activity in wild populations. Males are almost never found. At one locality where *falco*, *lucifer*, and *equinus* exist, *equinus* males are the commonest taken, *lucifer* males are relatively abundant, and *falco* males are never seen despite the fact that *falco* females are abundant.

DISCUSSION ON MATING BEHAVIOR

The ability of certain mosquitoes to mate in small cages, as compared with large ones, was observed by Roubaud (1930) as characterizing two independent biological races of *Culex pipiens*, namely, *molestus*, which would mate in a small space, and *pipiens*, which required a larger space.

Corradetti (1937) and Bates (1941) have shown great differences among the physiological races or species of the *Anopheles maculipennis* complex of Europe in the same type of mating behavior. Bates lists the different forms in the following order, according to their spatial requirements for mating, in the direction of smallest cage to largest cage: one *atroparvus*, two *labranchiae*, three *superpictus*, four *claviger*, five *sacharovi*, six *maculipennis*, and seven *subalpinus* and *messeae*. Of all these species, only *atroparvus* males did not need to swarm in order to mate, while neither *subalpinus* nor *messeae* would mate under any laboratory condition. Differences in light requirements for swarming and the character of mating were observed to be quantitative in character.

Boyd, Cain, and Mulrennan (1935) believe it possible that there had been a gradual adaptation to mating in smaller-sized cages in their colony of *A. quadrimaculatus*. Similar comment by Bates (1941) on *A. maculipennis* and *A. superpictus* has not seemed to reproduce these results. A colony of *A. superpictus* was kept in a cage for over 2 years with no wild material added; there was no discernible result in decreased or increased reproductive capacity. In the one case, it is possible that genetic variability was present to allow for genetic selection; in the other, this variability may have been absent.

The data of Bates cited above and those presented herein seem to indicate that the space requirements for different mosquito groups are not discrete ones, but are quantitative in character, such as might be produced by a multiple-factor inheritance. The hybridization data of Bates (1941) is indicative of a multiple-factor or quantitative-inheritance theory of behavior determination. Males of *A. maculipennis atroparvus*, crossed with females of *A. maculipennis maculipennis*, gave F_1 progeny which were very similar in behavior to those of another race, *labranchiae*. The latter is listed as intermediate in behavior between *atroparvus* and *maculipennis*.

Similar hybridization experiments made with *C. pipiens* by Tate and Vincent (1936) gave evidence that the same conclusions may be drawn, although their statements are difficult to interpret.

RATE OF DEVELOPMENT

The rate of development of an organism is a characteristic which is difficult to determine, owing to the inevitable fluctuations caused by environmental variations. Yet it is a characteristic of great value from a physiological point of view, for it is a manifestation of the speed of the metabolic processes in the organism.

In any comparison of growth rate between one species and another the actual speed determined as a biological characteristic has significance only when considered in relation with the particular environmental conditions used. In theory, it is essential that each species be provided with optimum conditions of growth, but this is often impossible in practice. Effective comparisons require experiments at various degrees of maxima and minima for the several environmental factors possible. Yet, a certain amount of valuable information can be extracted from development data obtained under "standard" laboratory conditions when the different species are grown at the same time, in the same place, and with the same food. It is this type of data that are to be presented here.

Several different species of *Haemagogus* from northwestern South America (Colombia) and *A. aegypti* have been grown in the laboratory for one or more generations from the wild. In several cases specific strains have been grown concurrently with identical environmental conditions imposed upon each. In these cases the development rates of the different species may be compared, one with the other. The temperatures in which this work was done averaged about 25°–27° C. It is clear that had higher or lower values been used in the experiments, the differential growth rates might have been somewhat different.

Growth rates at optimum temperatures probably greatly influence the ecologic distribution of a species, but the maximum and minimum temperatures are probably of equal or greater importance. This has been shown by Jobling (1938) in the forms *C. pipiens* and *C. molestus*. It appears that at most temperatures the growth rates of the two forms were the same but that at the lower ones the darker mosquito, *C. pipiens*, grew better. In the fly *Drosophila pseudoobscura* the fecundity of a northern race is higher than that of a southern one at lower temperatures, but the fecundity is reversed at the higher temperatures (Dobzhansky, 1935). In *Haemagogus*, however, 25° C. is within a few degrees of the average mean temperature preferred by each of the species, and so the range of variation may be unimportant.

The histograms represent five different sets of experiments on the growth rates of *Haemagogus* and *Aedes* (Fig. 1). Some of these are duplicated in different experiments; others may be represented but once. In two different experiments the same species may show different growth rates. This probably results from slight temperature alterations or changes in the larval food during the course of the experiment. Within a set, however, all conditions are comparable.

In the *Haemagogus* species it is clear that *equinus* is a faster-growing species than all the others tested. In all four of the experiments in which *equinus* is represented the mean and maximum speeds of larval and pupal development are more rapid than in any other species: *equinus* surpasses *splendens* and *falco* (Expt. 1), *falco* and *lucifer* (Expt. 2), *falco* (Expt. 4), and *falco*, *splendens*, and *A. aegypti* (Expt. 5).

The next most rapid species is *splen-*

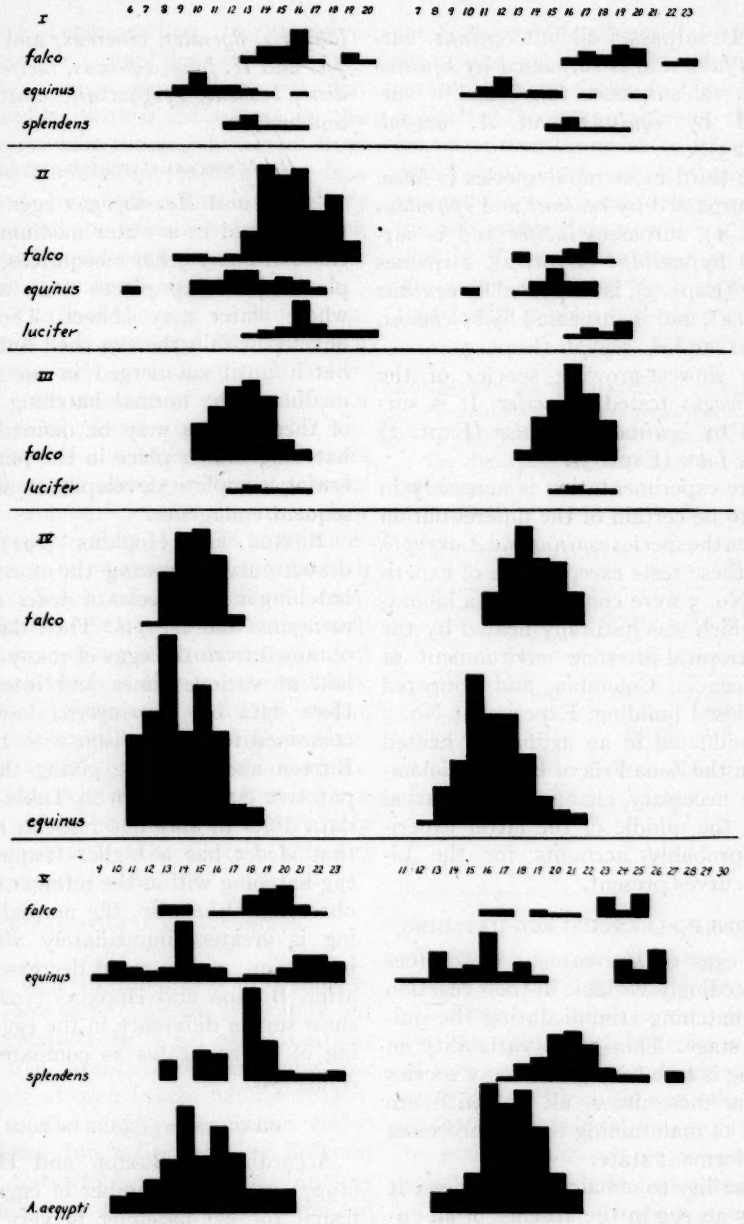

FIG. 1.—The comparative growth rates of the various *Haemagogus* species as represented by frequency diagrams. Histograms to the left represent the variation in growth covering the period from the hatched egg to the pupa; those to the right represent the period from the hatched egg to the adult. Times given are days. Growth rates of species are to be compared between sets grown under identical conditions; these sets are divided by lines.

dens. It surpasses all but *equinus:* surpasses *falco* and is surpassed by *equinus* (Expt. 1), surpasses *falco*, and is surpassed by *equinus* and *A. aegypti* (Expt. 5).

The third most rapid species is *falco*. It is surpassed by *equinus* and *splendens* (Expt. 1), surpasses *lucifer* and is surpassed by *equinus* (Expt. 2), surpasses *lucifer* (Expt. 3), is surpassed by *equinus* (Expt. 4), and is surpassed by *splendens*, *equinus*, and *A. aegypti* (Expt. 5).

The slowest-growing species of the *Haemagogus* tested is *lucifer*. It is surpassed by *equinus* and *falco* (Expt. 2) and by *falco* (Expt. 3).

More experimentation is necessary in order to be certain of the differentiation between the species *equinus* and *A. aegypti*.

All these tests except those of experiment No. 5 were conducted in a laboratory which was naturally heated by the lower-tropical-life-zone environment at Villavicencio, Colombia, and tempered by a closed building. Experiment No. 5 was conducted in an artificially heated room in the Zona Fría of Bogotá, Colombia. A necessary change in the larval diet in the middle of the latter experiment probably accounts for the bimodal curves present.

FACTORS INFLUENCING EGG-HATCHING

The eggs of *Haemagogus* mosquitoes are exceedingly variable in their reaction to egg-hatching stimuli during the quiescent stage. This great variability in hatching is true for a great many species of *Aedes* mosquitoes, all of which are capable of maintaining the life-processes in the dormant state.

The ability to remain in this quiescent stage as an egg in the absence of an enveloping water medium has been manifested in many mosquitoes of the *Aedes* group. These include: *A. aegypti*, *variegatus*, *kochi* var. *samoana*, *calopus*, *lateralis*, *dorsalis*, *cinereus*, and *varipalpus* and *H. falco*, *equinus*, *lucifer*, *splendens*, *boshelli*, *spegazzinii*, *anastasionis*, and *andinus*.

THE NORMAL HATCHING OF EGGS

Aedes and *Haemagogus* eggs are not usually laid in a water medium, as are those of many other mosquitoes, but are placed in damp places near water or where water may collect. The larvae develop within the egg shell but do not hatch until submerged in an aqueous medium. The normal hatching of eggs of these genera may be defined as the hatching taking place in the period following complete development and subsequent emergence.

Buxton and Hopkins (1927) have drawn curves showing the normal egg-hatching in two species of *Aedes*, namely, *variegatus* and *aegypti*.[2] Their data were obtained from the eggs of many females laid at various times and intermixed. Here data on *Haemagogus* have been combined for comparison with those of Buxton and Hopkins, giving the comparative curves shown in Table 2. The data differ in only one respect, namely, that *Aedes* has a higher frequency of egg-hatching within the number of days observed. Otherwise, the normal hatching is greatest immediately after the immersion, with a rapid decrease thereafter. Buxton and Hopkins (1927) also show such a difference in the egg-hatching of *A. variegatus* as compared with *A. aegypti*.

IRREGULAR HATCHING OF EGGS

According to Buxton and Hopkins (1927), when the number of eggs being tested for egg-hatching is very small, there are observed points of multiple

[2] The name *argenteus* was used by Buxton and Hopkins (1927) for the species referred to here as *aegypti*.

hatching corresponding to excessive emergence on certain days. The basis for this phenomenon was not clear, though all variables within the means of the techniques were thoroughly tested. Buxton and Hopkins (1927) had their data reviewed by statisticians, who have shown that the excessive hatching on these days could not be accounted for on grounds of random emergence alone.

The source of the eggs used in the experiments of Buxton and Hopkins was a large insectary (maintaining an indefinitely large number of mosquitoes) in which were placed containers for the deposition of the eggs. If one assumes that the eggs laid by different females of the same species are all identical or that the same females lay an equal number of eggs on successive days, then each batch of eggs may be considered comparable. However, it is possible that such large populations are heterogenous and that each batch of eggs is representative of different females—conditions leading to diverse results. When small numbers of eggs are studied, it follows, as necessary, that each batch of eggs from a given female should be studied separately from those of other females. Data of this sort are presented below.

THE INDIVIDUAL FEMALE FACTOR IN EGG-HATCHING

The eggs from various wild females of *H. falco* were collected separately but were retained in identical conditions with the intention of observing the hatching of each batch. After a reasonable "conditioning" period, which varied somewhat for collections at different times but which was identical in any one collection, the eggs were submerged in individual dishes, each containing an equal amount of the same leaf infusion.

Great differences were observed in egg-hatching among eggs laid by different females but subjected to identical environmental conditions. Some illustrative data are shown in Figures 2 and 3. Each of the units making up the histograms represents one batch of eggs from each female. Each unit is placed in the representative column corresponding to the percentage class to which it belongs; that is, if the egg-hatching in a batch was 0 per cent, the block representing that batch is placed in 0 per cent; if 7 per cent, the block is placed in the column 1–10 per cent; etc. The frequency of egg-hatching in any comparable batch is seen to vary from 0 to 100 per cent. With extreme variations of this sort between individual females, experiments which do not take into account the individual female factor may be rendered difficult of comparison by such a great uncontrolled variable.

TABLE 2

COMPARISON OF EGG-HATCHING FREQUENCY OF *A. variegatus* AND *Haemagogus* SPECIES FROM COMBINED DATA OF MANY EXPERIMENTS

The *Aedes* data are from Buxton and Hopkins (1927).

Day after immersion	*Aedes* (1,839 Eggs)		*Haemagogus* (5,665 Eggs)	
	No. of Eggs Hatched	Percentage Hatched	No. of Eggs Hatched	Percentage Hatched
1	949	62.1	1,161	22.7
2	258	16.9	268	4.7
3	67	4.4	118	2.1
4	54	3.5	.....	.....
5	32	2.1	.....	.....
6	14	0.9	.....	.....
7	28	1.8	.....	.....
8	18	1.2	.....	.....
9	5	0.3	.....	.....
10	12	0.8	.....	.....

The variation which a given female imparts to her eggs is apparent not only in the total number hatched but also in the number hatched on any particular day after immersion in the infusion. This

is shown in the Tables 3, 4, and 5 on *H. equinus*. Batch 365-2 in Table 3 had a hatching percentage of 94 per cent on the first day after immersion, whereas 365-4 had a hatching percentage of only 51 per cent—and this spread over 3 days. In 365-3, 14 per cent hatched on the third day after immersion; this was 78 per cent of the total hatched from that batch of eggs. It was common to have a large batch on the first day, as shown in Table 2 for the combined batches. A few selected variations in *H. falco* of the type included in the data summarized in Figure 3 are illustrated in Table 6. One set hatched its maximum on the first day; another set hatched none at all; and another set was characterized by scattered hatching, but primarily on the first day.

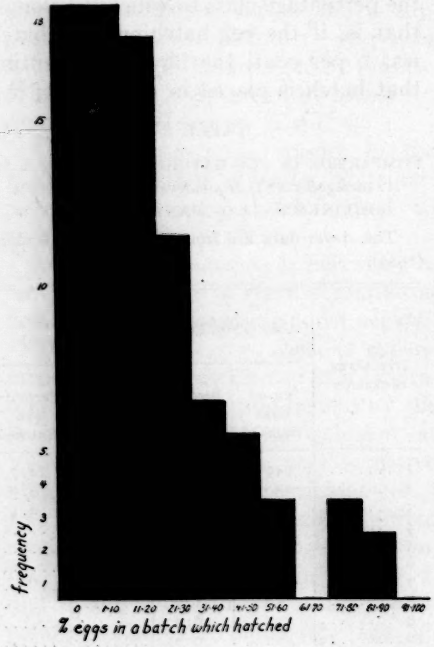

FIG. 2.—The frequency of *H. falco* eggs per female batch which hatched during a period of 6 days of immersion. These eggs are from wild females captured during the rainy season, during early September, 1943, in Villavicencio, Colombia. See also Table 8 for the data summarized in another way.

For comparison with this individual behavior some data of Buxton and Hopkins (1927) on *A. variegatus* may be examined (Table 7). Data of these investigators show that each batch of eggs from an unknown number of females reacted in very much the same way as would any particular batch of *Haemagogus* from a single female. They make this statement with regard to this aberrant hatching behavior: "One cannot at present explain these large random errors but one must never forget that they exist. . . . . " In explanation, it seems probable that, despite the large number of females exposed to the egg-laying site used by these authors, the eggs from any one day may have been laid by only one or a very few individu-

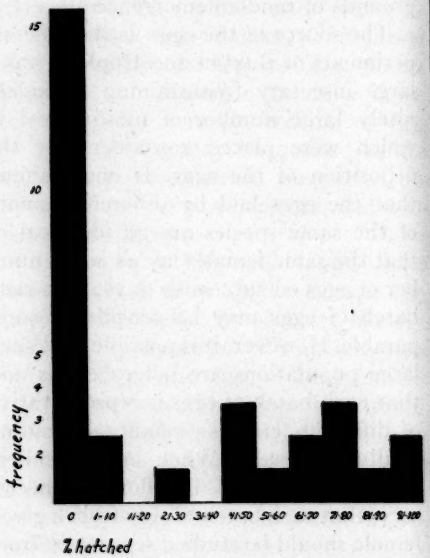

FIG. 3.—The frequency of *Haemagogus* eggs per female batch which hatched during a period of 5 days of immersion. These eggs are from wild females captured after the onset of the dry season, in late October, 1943, in Villavicencio, Colombia. See also Table 8 for the data summarized in a different way.

TABLE 3

EGG-HATCHING FREQUENCY IN INDIVIDUAL FEMALE BATCHES OF *H. equinus* FOR 5 DAYS

Batch 365-1 was split into two equal parts for comparison. All females were taken in the wild at the same place and time. In the totals the upper rows (1) represent the totals of all the eggs; the lower rows (2) represent the totals for the batches, not including those in which no eggs at all hatched.

Batch	No. of Eggs	Day after Immersion						Percentage of Total Eggs Hatched
		1	2	3	4	5	Total	
365-1*a*	24	3	2	0	0	0	5	21 | 31
365-1*b*	24	7	2	1	0	0	10	42 /
365-2	50	47	0	0	0	0	47	94
365-3	49	4	0	14	0	0	18	37
365-4	51	16	7	0	3	0	26	51
365-9	17	12	0	0	0	0	12	71
365-11	20	0	0	0	0	0	0	0
365-12	45	0	4	22	5	0	31	69
365-14	38	0	0	1	0	0	1	3
365-16	3	0	2	0	0	0	2	67
365-19	88	19	25	5	0	0	49	56
365-23	18	7	0	0	0	0	7	39
365-25	30	11	1	5	6	0	23	77
Total (1)	457	126	43	48	14	0	231	50.7
Total (2)	437	.....	.....	.....	.....	.....	.....	53.0
Daily totals:								
Percentage of eggs hatched (1)	.....	27.5	9.4	10.5	3.1	0	50.7	.....
Percentage of eggs hatched (2)	.....	28.1	9.8	11.0	3.2	0	53.0	.....

TABLE 4

EGG-HATCHING FREQUENCY IN INDIVIDUAL SISTER BATCHES OF *H. equinus*

These sisters were mated *en masse* with their brothers, being adults from 365-2, shown in Table 3.

Batch	No. of Eggs	Day after Immersion										Percentage of Total Eggs Hatched
		1	2	3	4	5	6	7	8	9	Total	
1	45	11	0	0	.....	0	0	.....	0	.....	11	24
2	46	25	1	0	.....	0	0	.....	0	.....	26	57
3	32	13	4	0	.....	0	0	.....	0	.....	17	53
4	45	8	2	0	.....	10	0	.....	4	0	24	53
5	52	18	0	0	.....	2	0	.....	1	0	21	40
6	69	50	2	0	.....	7	0	.....	0	.....	59	85
7	52	0	0	0	.....	0	0	.....	0	.....	0	0
8	37	2	8	0	.....	0	0	.....	0	.....	10	27
9	57	1	1	0	.....	2	0	.....	5	0	9	16
10	28	10	1	0	.....	0	0	.....	0	.....	11	39
Total (1)	463	138	19	0	.....	21	0	.....	10	0	188	40.6
Total (2)	411	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	45.8
Daily totals:												
Percentage of eggs hatched (1)	.....	30.5	4.1	0	.....	4.5	0	.....	2.2	0	40.6	.....
Percentage of eggs hatched (2)	.....	33.6	4.6	0	.....	5.1	0	.....	2.4	0	45.8	.....

als. Should this be true, then the "erratic" behavior described by these authors would be explicable.

THE EFFECT OF SOME UNKNOWN BUT PROBABLY SEASONAL INFLUENCES ON EGG-HATCHING

Diapause or developmental quiescence in animals of the temperate zones of the earth is usually correlated with climatic change. In the tropical regions yearly change in climate is largely the difference between dry season and wet season; any diapause would seem useful for spanning some such unfavorable period only. The eggs derived from female mosquitoes collected at different seasons of the year show differences in the percentage of eggs which hatch.

The data given in Figures 2 and 3 and Table 8 illustrate the differences in hatching between *H. falco* eggs having their source in wild females taken in different seasons. The females which laid the eggs examined in Figure 2 were caught in the month of September, before the onset of the dry season. Those in Figure 3 were caught in late October, well into the tropical dry season, when adults were much more difficult to obtain.

The primary difference between the two histograms seems to be that in Figure 3, if the eggs of any female

TABLE 5

EGG-HATCHING FREQUENCY IN INDIVIDUAL SISTER BATCHES OF *H. equinus*

These females were mated with their brothers *en masse*, as in Table 4; they were the adults from the female 365-12 shown in Table 3.

Batch	No. of Eggs	Day after Immersion			Percentage of Eggs Hatched
		1	2	Total	
1	30	0	0	0	0
2	32	4	3	7	22
3	4	0	0	0	0
4	22	0	0	0	0
5	3	0	0	0	0
6	31	16	0	16	52
7	18	0	0	0	0
8	11	2	0	2	19
9	21	0	0	0	0
10	32	0	0	0	0
11	23	0	0	0	0
Total (1)	227	22	3	25	11.0
Total (2)	74	.....	.....	.....	33.8
Daily totals:					
Percentage of eggs hatched (1)	.....	9.7	1.3	11.0	.....
Percentage of eggs hatched (2)	.....	29.8	4.1	33.8	.....

TABLE 6

HATCHING BEHAVIOR OF SOME SELECTED BATCHES OF *H. falco* EGGS FROM INDIVIDUAL FEMALES TREATED EXACTLY ALIKE IN ORDER TO ILLUSTRATE THE WIDE VARIABILITY IN TYPE OF HATCHING RESPONSE TO THE IMMERSION STIMULUS

These data are included in Figure 3.

Batch	No. of Eggs	Day after Immersion						Percentage of Eggs Hatched
		1	2	3	4	5	Total	
1	37	32	0	0	0	0	32	86
2	26	0	0	0	0	13	13	50
3	42	0	0	0	0	0	0	0
4	49	46	0	0	0	0	46	94
5	44	23	0	2	0	0	25	57

TABLE 7

THE HATCHING BEHAVIOR OF *A. variegatus* EGGS IN SEPARATE BATCHES BUT NOT NECESSARILY FROM THE SAME FEMALES, TO ILLUSTRATE THE SIMILARITY TO THE DATA OF *Haemagogus*

Compare with Tables 3, 4, 5, and 6. From the data of Buxton and Hopkins (1927).

No. of Eggs	Day after Immersion							
	1	2	3	4	5	6–10	Later	Total
15	8	0	3	0	2	0	0	13
22	20	0	0	0	0	0	0	20
13	0	0	0	0	0	10	0	10
14	0	1	0	0	1	4	2	8

hatched at all, they hatched nearly completely but that fewer hatched all together, because fewer individual batches hatched (50 per cent of the total batches). In Figure 2, on the other hand, a much higher percentage of batches hatched (78.3 per cent), but relatively few eggs in any one lot, so that the actual frequency of eggs hatched was lower (from 18.4 per cent in the latter to 31.2 per cent in the former) (Table 8). The ratio between eggs hatched, not including those in which none at all hatched, and the batches in which one or more hatched is important. In Figure 2 this is 22.2:78.3, as compared with 51.0:50.0 in Figure 3. These great differences in one species from the same place but collected at different times may be compared with three different independent species collected at the same place at the same time. This would serve as a natural test of the influence of the environment in preregulating the conditioning of the eggs for hatching.

TABLE 8

SUMMARY OF THE DATA UPON WHICH THE HISTOGRAMS (FIGS. 2 AND 3) ARE BASED SHOWING PERTINENT COMPARATIVE INFORMATION ON THE HATCHING OF EGGS OBTAINED FROM FEMALES TAKEN IN THE WILD AT DIFFERENT TIMES

Characteristic	Wet Season (=Fig. 2)	Dry Season (=Fig. 3)
A. Total number of eggs	2,550	741
B. Total number of egg batches (each from a single female)	83	30
C. Average number of eggs per batch (A÷B)	30.7	24.7
D. Number of batches in which one or more eggs hatched	65	15
E. Percentage of total batches (D÷B)	78.3	50.0
F. Number of eggs in batches under D	2,118	455
G. Average number of eggs per batch (F÷D)	32.6	30.3
H. Percentage of total eggs which hatched	18.4	31.2
I. Percentage of eggs which hatched in those batches in which at least one or more egg hatched	22.2	51.0

Females of three different species of *Haemagogus*, namely, *falco*, *equinus*, and *lucifer*, were obtained from a locality where all three were known to exist. As it was very difficult to be certain of identification of these species in the female sex, no attempt was made to identify them until the completion of the experiment. Eggs were obtained from each of these females individually and treated exactly alike.

The data on these three species (Fig. 4) show an interesting correlation in the character of egg-hatching. This is especially marked between *falco* and *lucifer*, while insufficient numbers make it impossible to determine if *equinus* conforms to the same pattern. In any event, there is a much better relation between the three species taken at the same time than there is between groups of one species taken at different times (Figs. 2 and 3), although the great individual female differences in hatching are still apparent. There seems to be no way of accounting for this similarity except to assume that the environment has affected the females in such a way that the eggs hatch in the same manner.

THE INFLUENCE OF STRAIN SELECTION

If it is true that some condition of a hereditary nature, which affects adults, influences the hatching nature of eggs

laid by these adults, then such a condition would become apparent by strain selection. Since it was possible to carry on laboratory breeding at this time only with *H. equinus*, the following information applies to this species only.

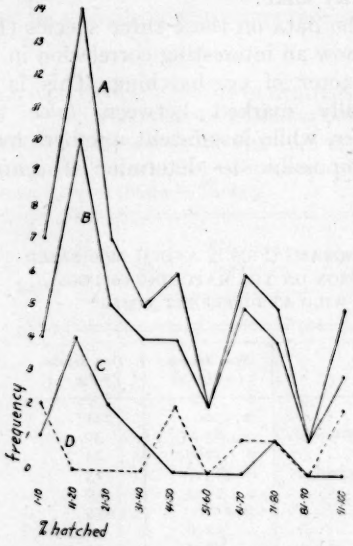

FIG. 4.—The frequency of *H. falco*, *equinus*, and *lucifer* eggs per female which hatched during an equal period of immersion. These eggs are from wild females captured at the same time in one locality, in the Magdalena Valley, Colombia, where all three species occurred together. The upper curve (*A*) represents the combined data of all three species. The second curve (*B*) represents the data on *falco*. The third curve (*C*) represents that on *lucifer*, while the fourth curve (*D*, dotted) represents the data on *equinus*. The similarity in the curves for *falco* and *lucifer* is very striking and appears to indicate the action of some general factors influencing these species where they exist at the same place, at the same time, in contradistinction to the differences between the same species when tested at different times (Figs. 2 and 3).

The data on egg-hatching from a set of wild females are shown in Table 3, each individual female batch being considered separately. The results generally indicate a good percentage of hatchings, the batches from some females being very good (365-25 or 365-12), others being very poor (365-14 or 365-11), and still others being intermediate (365-23 or 365-3). The combined egg-hatching (total) for the daily frequency curve shows about the same results as for the normal (Table 2), although that for the third day is a bit abnormally high.

In one particular example, 365-2, there was a nearly complete egg-hatching on the first day of emergence. In another, 365-12, hatching was delayed and scattered, together with a smaller total percentage. Larvae from eggs of these batches were used in *inter se* crosses, the results of which are given in Tables 4 and 5. The data in each of these latter tables represent the hatching results from eggs laid by sister females mated to brothers—perhaps the same individuals but not necessarily so. They represent selection with the genetic variability present in a given wild female and the sperm which she carried.

The eggs from the wild females (see Table 3) were subjected to identical conditions with one exception. That is, each batch was in a separate vial through the period of egg conditioning; therefore, the amount of drying to which each was subjected could not have been equal, though none was dried thoroughly. Batch 365-12, however, was becoming visibly dry; and this may, therefore, account for the differential results present in its egg-hatching. After this observation subsequent eggs of a given set were always placed in conjunction within a single covered Petri dish upon damp cotton. Batches as represented in Tables 4 and 5, therefore, have had equal external conditions imposed.

In the egg batches, shown in both Table 4 and Table 5, there is considerable fluctuation in hatching. Yet it is clear that the hatching has been greater in the set derived from 365-2, the set

which hatched the best in the preceding generation. With 365-2 females, the total egg-hatching was 40.6 per cent ($N = 463$); with 365-12 females, it was only 11.0 per cent ($N = 227$).

TABLE 9

THE COMPARATIVE HATCHING BEHAVIOR OF *H. falco, equinus,* AND *lucifer* EGGS WHICH HAVE BEEN TREATED IDENTICALLY AND WHOSE SOURCE WERE WILD FEMALES CAPTURED IN THE SAME PLACE AND AT THE SAME TIME

No. of Eggs	Day after Immersion				Percentage of Eggs Hatched
	1	2	3	Total	
	H. lucifer				
35	5	2	0	7	20
9	2	0	0	2	22
26	2	0	0	2	8
22	6	0	0	6	27
38	13	0	0	13	34
38	0	5	0	5	13
51	4	3	0	7	14
7	1	0	0	1	14
Total: 226	33	10	0	43	19
Daily percentage	14.6	4.4	0	19.0	
	H. equinus				
14	1	0	0	1	7
21	14	1	0	15	71
52	14	10	0	24	46
7	7	0	0	7	100
22	14	0	0	14	64
11	1	0	0	1	9
11	4	0	1	5	45
32	0	0	3	3	94
Total: 170	55	11	4	70	41.2
Daily percentage	32.4	6.5	1.5	41.2	

Whether this great difference is caused by genetic conditions present in the strain or by the effects of the partial drying given the eggs in the preceding generation is not clear. Nevertheless, the effects must have been passed from the eggs to the mother and then to the egg progeny of the following generation. That they may well be genetic is illustrated by Table 3, 365-1*a* and 365-1*b*, where a set is split and gives similar results.

TABLE 9—*Continued*

No. of Eggs	Day after Immersion				Percentage of Eggs Hatched
	1	2	3	Total	
	H. falco				
25	4	4	0	8	32
26	10	10	0	20	77
13	1	1	0	2	15
15	7	3	0	10	67
12	5	3	0	8	67
13	11	0	0	11	85
8	4	1	0	5	63
34	4	17	0	21	62
9	1	1	0	2	21
10	3	2	0	5	50
22	8	4	0	12	55
20	4	1	0	5	25
12	11	0	0	11	92
18	10	0	0	10	56
7	1	0	4	5	71
8	4	0	0	4	50
17	3	0	0	3	18
25	3	2	0	5	20
18	3	3	0	6	33
16	0	2	0	2	13
3	0	0	1	1	33
17	2	0	0	2	12
12	0	3	0	3	25
10	0	1	0	1	10
24	0	0	3	3	13
Total: 394	99	59	8	165	41.9
Daily percentage	25.5	14.7	2.1	41.9	

SPECIFIC DIFFERENCES IN *Haemagogus* SPECIES

Different species within the same genus of *Aedes* and *Haemagogus* react differently in regard to percentage of eggs which hatch under similar stimuli. Thus Buxton and Hopkins (1927) have shown a definite difference of several per cent between *A. aegypti and A. variegatus*. Data given in Table 9 indicate differences between *Haemagogus* species reacting under the same stimulus.

Haemagogus lucifer has a decidedly poorer egg-hatching than either *H. equinus* or *H. falco*, the latter two appearing to be essentially alike: *H. lucifer*, 19.0 per cent (N = 226); *H. equinus*, 41.2 per cent (N = 170); and *H. falco*, 41.9 per cent (N = 394). These differences reveal that the egg-hatching frequency is under genetic control and is correlated with the species differences.

RELATION OF THE NUMBER OF EGGS LAID BY A GIVEN FEMALE TO THE FREQUENCY OF EGGS WHICH HATCH

The data have been calculated in various ways for the purpose of testing a suggestion that it might be the older or more poorly fed females which lay the eggs having the poor frequencies of hatching.

First, all the data on *Haemagogus* were divided into those egg batches in which 50 per cent or more hatched and those in which 49 per cent or less hatched, checking for each the number of eggs of that batch. This was assuming that the older or more poorly fed females would lay fewer eggs. These data are shown in Table 10.

There is an average number of 27.3 eggs per female batch in those having 50 per cent or more hatching (N = 1,230), and 28.2 eggs per female in the remainder (N = 3,392). Clearly, these are essentially the same and show no differential correlation.

Those batches in which no eggs at all hatched have been included with those in which less than 50 per cent hatched. Now, if these batches were infertile, the data would not be accurate. Consequently, it would be desirable to obtain the figure of eggs per female excluding these. The number obtained in this way is 34.3 eggs per female (N = 1,855), which is considerably more than before. The number obtained for batches in which no eggs hatched is 24.4 (N = 1,537), or considerably less.

With other things being equal, it might be expected that smaller batches would show a higher frequency of batches with no hatching than do larger ones, since a lower percentage of hatching would not be apparent in very small batches. There is no reason to suspect that the difference shown is anything more than this. On the other hand, were all batches of eggs fertile, the removal of batches in which no eggs hatched would result in a rise in the mean number of eggs in the remainder. This rise is shown in the mean of 34.3 shown in these data. Therefore, it may be assumed that nearly all batches are fertile and that there is evidently nothing to support a correlation between number of eggs per female and the frequency of eggs which hatch.

A correlation of this sort is evident, however, in the specific data given for the inbred lines of *H. equinus* (Tables 4 and 5), even though in the general data it is absent. The females derived from 365-2 (Table 4) laid more eggs than the females derived from 365-12

TABLE 10

THE RESULTS OF CORRELATING THE NUMBER OF EGGS LAID BY A FEMALE AND THE PERCENTAGE OF EGGS LAID BY IT WHICH HATCHED

	No. of Batches	No. of Eggs	Mean No. of Eggs per Female Batch
Batches of which 50 per cent or more hatched	45	1,230	27.3
Batches of which less than 50 per cent hatched	120	3,392	28.2
Batches in which less than 50 per cent hatched but excluding those in which none hatched	57	1,855	34.3
Batches in which no eggs hatched	63	1,537	24.4

(Table 5). These averaged 46.3 eggs per female for the former, as compared with 22.7 for the latter. Egg-hatching frequency in the former was 40.6 per cent, and in the latter 11.0 per cent; or, with those batches in which no eggs hatched removed from the total, 45.8 per cent for the former (365-2) and 33.8 per cent for the latter (365-12). A relation of this sort might be accounted for only by assuming an influence upon female egg production by partial drying of the eggs from which the females are to emerge, by a genetic line having these qualities, or by poor fertility in the crosses.

DISCUSSION OF FACTORS INVOLVED IN THE VARIABLE EGG-HATCHING

The discussion of egg-hatching phenomena is, by the nature of the problem, divisible into two parts. One part covers the type of phenomenon discussed above, namely, factors influencing or controlling the capacity of eggs to hatch when subjected to a stimulus. The other part covers the physiological mechanism by which the eggs are enabled to remain in a quiescent stage. The former part will be discussed here; of the latter, only a brief mention will be made, since but little information is available.

The frequency of eggs hatching after a stimulus is clearly influenced by the genetic or hereditary possibilities which they have, as determined by the eggs themselves or by the female which laid them. Secondarily, the genetically determined frequency is influenced by the environmental conditions to which the eggs or the female adult have been subjected.

The existence of a heritable condition is clearly shown by the differences between the species. It is also shown by the tremendous differences between individuals of the same species when these individuals have had identical conditions throughout their lives. It is inconceivable that all this variation is environmental. The effects of genetic selection for one generation are inconclusive on this question but suggest a possible selective influence toward ability or inability to hatch easily. In addition to this information, Shannon and Putnam (1934) mention that the resistance to egg-hatching stimuli may be peculiar to certain strains of *A. aegypti*, judging from a Greek strain; but data are absent.

The effects of the environment are conclusive in so far as the actual presence of an influence is concerned. In our consideration we have been interested only in the effects applied before the actual eggs themselves are obtained. The effects of direct environmental manipulation upon the eggs are a different problem and will be considered below.

Between the time of egg-laying and the time when the eggs are first capable of hatching, there must be an embryonic period when the larvae are developing. This period is manifested as a necessary "conditioning" period by the practical mosquito-breeder, as is shown by the following data.

Shannon and Putnam (1934) have presented data which give the most complete record of the phenomenon in this group of mosquitoes. They have used *A. aegypti*, but the results are comparable to other similar mosquitoes too. The data are of the following type. Eggs were collected at given mean hourly intervals after laying, kept in moist conditions for a definite period of time for "conditioning," and then immersed in infusion for the purpose of recording the time interval after immersion when the eggs are capable of hatching. In this way it is possible to construct curves showing the relation between conditioning time and hatching time, as shown

in Figure 5. These curves are based upon tabular data supplied by Shannon and Putnam (1934), collected at a reasonably constant temperature.

Figure 5 was constructed by placing the "conditioning" time (the time from egg-laying to immersion) on the vertical scale and the "additional" time (the time from immersion to egg-hatching) on the horizontal scale. On the latter scale is also given, in separate curves, the time interval between first and last hatchings, the total number of elapsed hours up to first egg-hatching, and the total number of hours accumulated until the last eggs have hatched.

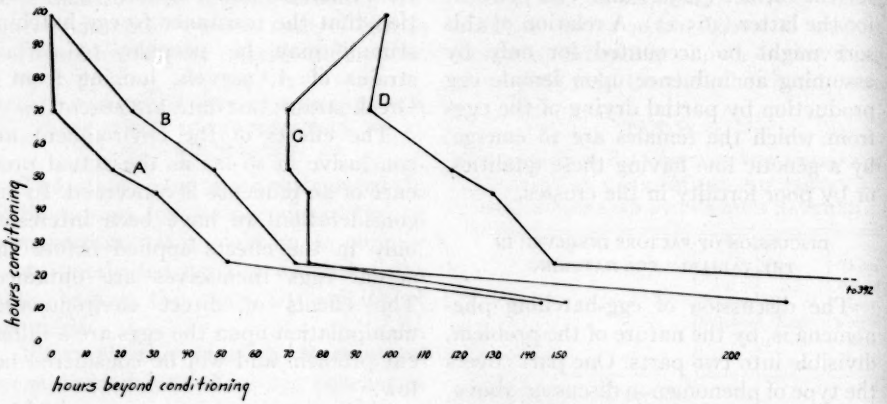

FIG. 5.—Curves showing the relation between length of time of egg-conditioning and length and character of egg-hatching after immersion. For example, if eggs are conditioned or ripened for 52 hours (as shown in vertical time), then there is an additional period of 18 hours necessary before the eggs hatch (as shown by horizontal time). Such points as have been determined are shown on the curves.

Curve *A* represents the time necessary for egg-hatching after given intervals of conditioning. It shows the inverse relation between egg-conditioning time and after-immersion time up to 70 hours maximum.

Curve *B* represents the number of hours' interval between the time of first egg-hatching and the last egg-hatching after a certain number of hours of conditioning. This curve illustrates an inverse relation between time of conditioning and spread of egg-hatching after immersion up to 99 hours of conditioning.

Curve *C* represents the total number of accumulated hours (hours of conditioning plus after-immersion time) which have elapsed up to the time the first eggs hatch at any given period of egg-conditioning. Here is illustrated the inverse relation of total time elapsed to conditioning time up to about 25 hours of conditioning, then no great change to 70 hours, and then a direct relation of increase in total elapsed time to conditioning time.

Curve *D* represents the total number of hours of accumulated time (as in *C*) which have elapsed up through the time the last eggs hatch at any given period of egg-conditioning. It illustrates an inverse relation of total time elapsed to conditioning time up to 70 hours of conditioning and thereafter a direct relation. Thus there is no advantage in conditioning eggs beyond 70 hours in *A. aegypti*.

(Data from table by Shannon and Putnam, 1934.)

If it is assumed that the period from egg-laying until the time when immersion has the effect of causing some egg-hatching is the period of egg development, the results may be described in this way: When immersion of the eggs takes place before the larval development is completed, the stimulus of immersion in a liquid medium has no effect, or a delayed one, on subsequent egg-hatching. The obvious influence is one of delaying the hatching longer, the earlier the eggs are immersed (curve *A*, Fig. 5). This relation holds up to a period of about 70 hours of conditioning, which persumably is the period when

most eggs have reached maturity and additional conditioning is of no advantage.

The effect of this delay in egg-hatching (or development) is shown by the curve *C*. Less than 24 hours of conditioning greatly delays egg-ripening, while an interval of between 24 and 70 hours has little effect; at least the combined time of egg-conditioning and time required for hatching is not different between 24 and 70 hours. As shown by curve *B*, however, the slowest eggs in a batch appear not to be fully developed at 70 hours, and it is not until 99 hours that all are ready for hatching. The interval between the first and the last hatching of eggs increases greatly, the earlier they are immersed in infusion. Thus, the total number of hours elapsed for complete egg-hatching can be shown by a curve *D*, with an inverse relation between the conditioning time of the egg and the total time elapsed up through 70 hours of conditioning and then a direct relation thereafter. For practical purposes, where speed of generation turnover is desired, it is best that the eggs be conditioned not longer nor less than about 70-99 hours at the temperatures and with the species used by Shannon and Putnam (1934).

The delayed egg-hatching apparent when eggs have been insufficiently conditioned might be ascribed to either of two recognized factors. The first, which has been suggested by Shannon and Putnam (1934), is that development has been retarded. Another is that by premature immersion no subsequent stimulus is applied, so that eggs hatch at almost any later time. By either theory the increased spread of hatching in the insufficiently conditioned eggs could be accounted for, since greater differences of development would be detected by a slower development; also, lack of a sharp stimulus would be effective in producing the same result. The slower development of the eggs could be accounted for in the submerged eggs by the decreased availability of oxgyen in deep water, as compared with a thin film fully exposed to air surface.

Factors which might influence egg-hatching after conditioning are of many different sorts. There seem to be few possibilities open for experimentation which have not been covered already by Atkin and Bacot (1917), Bacot (1917), Young (1922–23), Roubaud (1929 and others), Gjullin *et al.* (1939, 1941), Buxton and Hopkins (1927), and Connell (1941).

The efforts of Buxton and Hopkins (1927) are worthy of special commendation. However, this is hardly the place to review the work in any more detail than to conclude that the stimulation effect of immersion in water is necessary for hatching and that, in addition, almost anything in the water will usually be better than water alone to restart larval activity, even if the substance is toxic to the eggs and larvae afterward. Buxton and Hopkins (1927), Shannon and Putnam (1934), and others have found that this effect is most pronounced when the eggs have been dried for some time before immersion. Gjullin, Hegarty, and Bollen (1941) feel that the important question is not the direct action of the materials themselves but their effects in altering the amount of dissolved oxygen, which is important. Let us inquire into the ways in which such things could act.

The *Aedes* type of egg diapause is of a class different from that of normal larval, pupal, or adult diapause. With respect to the latter, there is a considerable period of time elapsing before the animal's metabolism returns to normal, owing to the various physiological changes which

have occurred prior to the onset of the quiescent condition. The fact that the *Aedes* and *Haemagogus* larvae are immediately active within a few seconds of the activating stimulus when they are normally activated and released from the membranes and the chorion, whereas they are not active at all if released artificially, suggests the involvement of other factors. The case is similar to that of many eggs which cease development during the maturation divisions, continuing division only after the stimulation produced by the entrance of the sperm. In these cases similar stimulation, by pricking, etc., motivates the egg into continued cell division without fertilization.

In the *Aedes* egg, immersion in water immediately changes only two factors: the oxygen availability and the general pressure around the egg chorion. Since the larvae may be activated within seconds of immersion, it does not seem reasonable to suppose that the internal water content of the egg is substantially altered. Nevertheless, if the vitelline or later-developed egg membranes regulate the osmotic absorption by the egg, it is possible that such slight influence may be sufficient to bring the latent metabolic system of the larvae into action. It supplies a theory to account for internal expansion and chorion breakage, but the fundamental principles of activation remain as obscure as they are in the earlier embryonic or egg stages. Conditioning of the regulatory power of the egg membranes by the metabolic condition of the female through her ovaries seems quite plausible and may account for the delayed influence of the environmental effects.

SUMMARY

1. Laboratory observations on the mating behavior of South American *Haemagogus* species have been described. It is found that a difference in the area or space needed for the mating dance is present in these species. Including *A. aegypti* in the comparison, the observed order of size requirement for the mating (from small to large size) was as follows: (1) *A. aegypti*, (2) *H. splendens*, (3) *H. equinus*, (4) *H. lucifer* and *H. boshelli*, (5) *H. andinus*, (6) *H. spegazzinii*, and (7) *H. falco*. The latter three were not mated in captivity; the position was determined from activity only.

2. Activity of these mosquitoes is at least partly correlated with mating.

3. Comparisons are made between the species with respect to their mating tactics, that is, the comparative positions of the sexes during copulation and whether copulation is in flight or at rest.

4. This information is compared with similar data on *Anopheles* and *Culex;* in these genera the nature of mating behavior suggests quantitative inheritance of the phenomenon.

5. The rates of development of *H. equinus*, *A. aegypti*, *H. splendens*, *H. falco*, and *H. lucifer* are compared; it is found that the order of speed, from fastest to the slowest, is in the direction as given above, judging from direct and indirect comparisons. The actual time elapsed is variable, depending upon environmental conditions and upon inherent differences of each individual.

6. The species showing the most rapid rate, *H. equinus*, had a maximum speed of 6 days to the pupa and 7 days to the adult stage. The range of variation of a set grown in the same pan at the same time approximated the random variation expected around the mean of a normal curve.

7. The normal egg-hatching of *Haemagogus* mosquitoes is shown to be dif-

ferent only in degree, not in quality, from that of *Aedes*, as shown by Buxton and Hopkins (1927).

8. The type and frequency of egg-hatching in *Haemagogus* indicates a quality imposed through the female, which may be hereditary or environmental. The *Aedes* data of Buxton and Hopkins (1927) are found to lend themselves to the same considerations.

9. Seasonal or environmental influences on the female seem to alter the hatching of her eggs.

10. Genetic selection of strains within the species for egg-hatching differences seems to be highly possible. Interspecific strain differences are present in *Aedes* and *Haemagogus*.

11. There seems to be no correlation between the number of eggs laid by a female and the frequency of eggs which hatch in her progeny.

12. A distinction is drawn between two types of phenomena which must be considered in these problems: one covers the factors influencing the capacity of eggs to hatch when subjected to a stimulus, and the other covers the physiological mechanism by which the eggs are enabled to remain in the quiescent stage.

13. The normal hatching phenomena have been discussed, and then the ways by which a breeder can take advantage of these in increasing generation turnover and number are advanced.

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URIC ACID FORMATION IN THE DEVELOPING EGG OF THE GRASSHOPPER, MELANOPLUS DIFFERENTIALIS[1]

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THAT the cleidoic or "closed type" of terrestrial egg produces uric acid as an end-point in its protein metabolism during its development seems well established (Needham, 1942). Such a type of nitrogen metabolism has been termed a "uricotelic" one. Among the organisms exhibiting this phenomenon, the insects are perhaps the classical example. Qualitative uric acid studies on this group are numerous, and the results obtained are to a great degree similar in that they invariably report its presence in the developing egg. Quantitative investigations on the insect egg during its entire development, however, seem few in number, and results obtained are, to a great degree, conflicting. The present paper deals with the formation and significance of uric acid in the egg of the grasshopper, *Melanoplus differentialis*, during its entire development under controlled and standardized conditions (Slifer, 1932).

MATERIAL AND METHODS

Results of extensive studies on the development of the egg of the grasshopper, *M. differentialis*, under controlled laboratory conditions, have made available much detailed information as to the chronological appearance of both structures and processes in the egg. Such biological material, therefore, is readily adaptable for a quantitative study of uric acid formation during embryonic development. All eggs were obtained, kept, and used under conditions previously noted (Slifer, 1932).

Eggs from pods of similar chronological ages were mixed and thoroughly washed in sterile tap water and then individually selected for uric acid determinations. The number of eggs employed was governed by their age and uric acid content and ranged from 400 to 50, the youngest eggs always being used in largest numbers. Excess tap water was removed and sterile distilled water added, after which the eggs were dried on filter paper. Extracts were prepared by thoroughly grinding the eggs in a sterile mortar and then adding 25 cc. of distilled water at 60° C. The contents of the mortar were quickly poured into a centrifuge tube and centrifuged for from 10 to 30 minutes. The thin lipid layer collecting on the surface of the centrifuged extract was pipetted off and discarded. The supernatant liquid was decanted off and used for uric acid determinations as outlined below.

During the preparation of extracts from eggs of different ages, certain marked changes in their physicochemical properties occur which may interfere with the successful carrying-out of colorimetric uric acid determinations. Eggs, up to 5 days at 25 °C., when ground and mixed with 25 cc. of distilled water at 60° C., produce a milky, opalescent extract, which requires long and strong centrifugation for clearing. In extracts

[1] This work was aided by a grant from the Rockefeller Foundation for research on the physiology of the normal cell.

of older eggs, a marked and well-formed precipitate is readily thrown down and firmly held in the centrifuge tube, allowing the clear supernatant fluid to be used at once. At about the seventeenth day of development at 25° C. the formation of a pink pigment (probably related to respiration?) becomes evident but causes little difficulty in the colorimetric determinations.

Uric acid was determined by the direct method of Benedict and Franke (1922), as modified by Christman and Ravwitch (1932). As indicated, little difficulty was encountered in obtaining comparable readings, except for eggs in early stages of development, up to 5 days at 25° C. The slight opalescence of these extracts tended to produce a greenish tinge in the treated samples, and this, in turn, interferes slightly in obtaining clear-cut end-points. With the aid of filters, however, most determinations in this region of the curves are considered as being approximately correct, as carefully checked by control experiments. Substances known to interfere with uric acid determinations by this method are practically absent from the egg extracts, since they are removed with the precipitate thrown down during centrifugation. The amounts of distilled water (at 60° C.) used in preparing the extracts were always in excess, so that all soluble materials were taken into solution. All determinations were carried out in triplicate, and averages were obtained for different lots of eggs of similar ages. Results are expressed in terms of milligrams of uric acid per egg.

RESULTS

Results of experiments have been summarized and expressed graphically in Figure 1. Upon examination of this figure, one notes a very marked increase in the uric acid content of the egg throughout the course of its development, beginning at an approximate value of 0.0004 mg. per egg and ending with hatching at 0.035 mg. per egg. These increases are especially striking during the prediapause and postdiapause phases and correspond, therefore, to the periods during which active development (cell

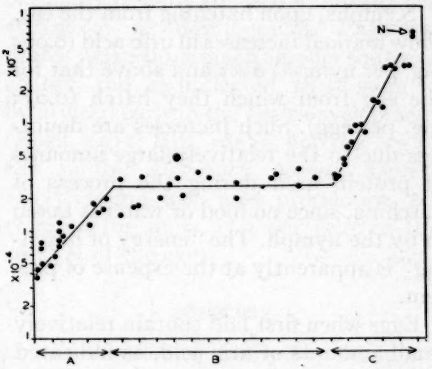

FIG. 1.—Shows amounts of uric acid produced in the egg of the grasshopper, *M. differentialis*, during its entire development at 25° C. *Ordinate:* uric acid, milligrams per egg expressed on semilogarithmic scale. *Abscissa:* time in days, 5-day intervals indicated, beginning at laying of eggs; *A*, prediapause; *B*, diapause; *C*, postdiapause. Each point on curve represents average obtained for at least three lots of experimental eggs. Letter *N* represents average uric acid values for nymphs just after hatching. Postdiapause eggs obtained after treatment of diapause eggs at 5° C. for 2 months.

division and differentiation) of the embryo and associated structures takes place. During the "blocked," or diapause, phase no significant changes in the uric acid content are apparent, even though the eggs remain in this condition at 25° C. for as long as 2–3 months. Variations in the uric acid values for different lots of eggs occur owing to slight differences in the rates at which they develop, but the general trends in the uric acid formation are clearly defined.

Upon examination of embryos re-

moved from the eggs, marked regions of uric acid or urate deposition are easily discernible, and these, in turn, parallel in abundance the relative amounts of uric acid found.

The general nature of the uric acid curve resembles that for the O_2 intake and the CO_2 output of the egg during its course of development (Bodine and Boell, 1936).

Nymphs, upon hatching from the egg, show marked increases in uric acid (0.065 mg. per nymph) over and above that for the egg from which they hatch (0.035 mg. per egg). Such increases are doubtless due to the relatively large amounts of protein used during the process of hatching, since no food or water is taken in by the nymph. The "energy of hatching" is apparently at the expense of protein.

Eggs when first laid contain relatively small amounts of uric acid, as indicated in Figure 1. This amount (approximately 0.0004 mg. per egg) is doubtless that obtained from maternal body fluids during the course of egg formation.

DISCUSSION

As far as can be ascertained, the present results seem to be the only available ones for uric acid formation in an insect egg, raised under standard laboratory conditions and throughout the entire course of its development. That the grasshopper egg is a true "closed" or cleidoic one, maintaining a uricotelic type of metabolism throughout its development, seems well established.

Comparisons between the amounts of uric acid in the egg of the grasshopper with those for other insects are extremely difficult because of the lack of sufficient data on the periods of development for which determinations were made. Complete references to such data are noted by Needham (1942) in discussions on the biochemical changes during development. Among the forms of insects upon which much work seems to have been carried out is the silkworm. Determinations of uric acid in the egg of this form have been repeatedly carried out, and among more recent investigators the results of Manunta (1937) seem of peculiar interest. This author found the uric acid content of the egg to be as high as 6.0 mg/gm of egg but noted no marked changes during the development of the egg and even attributed the total uric acid present to that deposited in the egg by the maternal organism.

The kinds and relative amounts of various materials burned or utilized during embryonic development have been questions of much interest and speculation, and especially so in respect to typical cleidoic eggs. It has usually been assumed that the terrestrial cleidoic egg consumes large amounts of fat and much smaller amounts of protein and carbohydrate during development (Needham, 1942). On the other hand, aquatic eggs, those especially dependent upon their environment, are thought to burn more protein than fat. These differences in the food materials consumed by the two types of eggs are usually attributed to the fact that aquatic eggs are able to take in and get rid of liquid substances, while terrestrial ones are faced largely with the difficult problem of conserving water and getting rid of excretory products, and especially the nitrogen compounds. According to Needham (1942), approximately 80 per cent of the total material burned in the development of the terrestrial egg is fat, while for the aquatic egg it is but 32 per cent. For protein, the terrestrial egg uses approximately 6.8 per cent, while the aquatic type consumes 62 per cent. For the

grasshopper egg, as a typical terrestrial egg, we find a burning of approximately 72.5 per cent fat (Slifer, 1930) during the course of its development. That small amounts of carbohydrate are used is indicated from the R.Q. curves of Boell (1935) and the more detailed study of Hill (1943). Oxygen consumption and carbon dioxide curves for the developing grasshopper egg have been shown to follow definite cycles, which, in turn, are related to the cellular activity of the developing egg (Bodine, 1932*a*). The total nitrogen content of the grasshopper egg has been shown to remain practically constant throughout the entire developmental period (Trowbridge and Bodine, 1940), indicating no loss or gain in nitrogen from the environment. "The rate of oxygen consumption per milligram of embryo nitrogen rises steadily in prediapause, but drops rapidly at the onset of diapause, the diapause level being 1.7 mm.$^3O_2$/mg. N. As soon as development resumes, the rate rises again until, on the fifth day, it reaches 20 mm.$^3O_2$/mg. N, where it remains practically constant throughout the remainder of the embryonic period" (Trowbridge and Bodine, 1940). The average total nitrogen per egg throughout development is approximately 0.18 mg. (Trowbridge and Bodine, 1940). The highest value found for uric acid in the developing grasshopper egg is approximately 0.035 mg. Assuming the uric acid nitrogen to represent the amounts of protein compounds transformed, we find that approximately 6.6 per cent of the total protein is used during the course of the egg's development. It should be pointed out, however, that undoubtedly a small amount of nitrogen used may be concerned with "wear and tear," as well as with the formation and digestion of the egg cuticle. This amount would, at most, be relatively insignificant in such considerations. It thus appears that, for the grasshopper egg, approximately 72.5 per cent of fat (Slifer, 1930) and 6.6 per cent of protein originally present are used up during the course of its development. Carbohydrates make up a much smaller source of energy, and, as indicated, these seem to be utilized early in the egg's development (Boell, 1935; Hill, 1943). For the egg of the moth, *Antheraca pernyi*, Leifert (1935) found uric acid production accounted for some 33.3 per cent of the total N of the egg.

SUMMARY

1. A detailed, quantitative study has been made of the uric acid production during the complete development of the egg of the grasshopper, *M. differentialis*.

2. A marked increase in uric acid occurs during the developmental phases, ranging from 0.0004 mg. per egg at the beginning and 0.005 mg. per egg at hatching. During diapause, or "block," no marked changes in the concentration of uric acid in the egg occur.

3. The general trends of the uric acid curve are similar to those for the O_2 intake and CO_2 output of the egg.

4. Evidence is presented suggesting that, during active development of the egg, approximately 6.6 per cent of the initial protein is transformed or broken down.

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MODIFICATIONS OF SAND-DOLLAR DEVELOPMENT BY EXPOSURE TO LITHIUM CHLORIDE AND SODIUM THIOCYANATE BEFORE AND AFTER FERTILIZATION[1]

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MANY substances have been found which modify the pattern of development in the echinoderm egg. Such substances as NaCl, $HgCl_2$, $CuSO_4$, KCN, NiCl, neutral red, methylene blue, auxin, glycogen, chloroform, X-rays, CO, CO_2, NaCNS, pilocarpine, lithium, etc., when applied after fertilization, produce various modifications, depending on the nature of the agent, the concentration, the length of the exposure period, and the time of exposure (see Child, 1941). Among the common types of modifications with postfertilization treatment are (1) inhibition of ectoderm with increase of entoderm as evaginated gut; (2) increase in mesenchyme or internal cells; (3) inhibition of ventral surface, producing various angles to anal arms, depending on the degree of inhibition and recovery; (4) inhibition of oral lobe and arms; (5) anomalous distribution of mesenchyme and skeleton; and (6) modifications in coelomic pouches.

Many of these modifications are the results of inhibition during critical periods, inhibition of regions of higher susceptibility, differential recovery, differential conditioning, etc. Child suggests that the large evaginated entoderm oc-

[1] The experimental data of this paper were obtained at the Hopkins Marine Station, Pacific Grove, California, during the summer of 1941. The writer wishes to express his gratitude to the staff of this station for many courtesies during the progress of the work. To Dr. C. M. Child the writer is especially indebted for valuable criticisms during the preparation of the manuscript.

curring after treatment with many different agents may be a differential modification, depending on nonspecific alterations of a primarily quantitative gradient pattern. From his extensive work with the effects of lithium on sand-dollar development he points out that the large evaginated entoderm is largely presumptive ectoderm which has been lowered by the inhibitory action of lithium to the physiological level of entoderm. This increase in entoderm has been termed by various workers "entodermization."

Lindahl (1933, 1936) found that varying degrees of "ectodermization," or the development of ectoderm at the expense of entoderm, could be obtained in sea-urchin larvae by the treatment of eggs with a number of agents before fertilization. Of the agents used, NaCNS in Ca-free sea water seemed most effective. Rulon (1941) obtained similar results with sand-dollar eggs with similar treatment, that is, when treated before fertilization with NaCNS in Ca-free sea water. It was also found that, while the thiocyanate before fertilization ectodermized the larvae, it *entodermized* the larvae when used for varying intervals following fertilization. Lindahl (1936) also found that lithium in Ca-free sea water before fertilization produced entodermization in contrast to the action of thiocyanate.

The present investigation, with sand-dollar eggs as the experimental material, deals with (1) experiments involving the use of lithium before fertilization and (2) experiments involving a double treatment in which thiocyanate was used before, and lithium after, fertilization.

MATERIAL AND METHODS

All work was done on the eggs of the sand dollar, *Dendraster excentricus*. Mature females were opened, and only the ripe red eggs exuding from the ovaries were used. These were washed several times in sea water before the experiment was begun. After the proper exposure to the test solutions, the eggs were washed and fertilized in normal sea water by adding a few drops of sperm suspension. Observations on the developing larvae were made at regular intervals up to 96 hours. The temperature ranged around 17° C. All experiments were performed with approximately one hundred and fifty eggs in 150–200 cc. of solution in fingerbowls. During the intervals between observations, the material was kept covered on the water table. Several controls were always run along with the tests. All solutions were osmotically controlled.

At the crest of the breeding season (around July 15) unfertilized eggs, removed from the ovary and allowed to stand in sea water, were still fertilizable after 24 or more hours. During the last of the season the eggs seldom remained fertilizable for longer than 12 hours. Repeated experiments showed that the period of fertility in *Dendraster* eggs could be extended by placing them in a sea-water solution of 1.0 per cent ethyl alcohol.[2] Since strong solutions of lithium in Ca-free sea water were found to shorten the period of fertility, alcohol was usually added to insure sufficiently long exposure in experiments in which conditions called for the use of lithium in Ca-free sea water before fertilization.

EXPERIMENTAL DATA

I. THE EFFECT OF LITHIUM TREATMENT BEFORE FERTILIZATION ON THE DEVELOPMENT OF SAND-DOLLAR EGGS

Lithium chloride in normal sea water had little effect on unfertilized eggs at concentrations which permitted fertiliza-

[2] Experiments by Schechter (1937), Whitaker (1937), and others have demonstrated that the fertile period of various marine eggs can be lengthened through the use of low-Ca sea water, ethyl alcohol, and other substances.

tion and development. Experiments with lithium in Ca-free sea water were inconclusive, although there was some evidence of entodermization at high concentrations. Unfertilized eggs exposed to 100 cc. of Ca-free sea water plus 100 cc. of 0.54 M LiCl for 12 hours could be fertilized in a few cases. In the development of these eggs the cleavage pattern was aberrant, blastulae were commonly broken down into their individual cells, and only a few grossly inhibited larvae appeared. When ethyl alcohol was added to the Ca-free lithium solutions, much better results were obtained.

Several series of experiments were run in which eggs were placed in the following solutions for 12 hours, after which they were washed several times in sea water and fertilized:

1.....Normal sea water
2.....Ca-free sea water[3]
3.....1 per cent alcoholic Ca-free sea water
4..... 50 cc. 0.54 M LiCl + 100 cc. Ca-free sea water + 1.5 cc. alcohol
5..... 60 cc. 0.54 M LiCl + 100 cc. Ca-free sea water + 1.6 cc. alcohol
6..... 70 cc. 0.54 M LiCl + 100 cc. Ca-free sea water + 1.7 cc. alcohol
7..... 80 cc. 0.54 M LiCl + 100 cc. Ca-free sea water + 1.8 cc. alcohol
8.....100 cc. 0.54 M LiCl + 100 cc. Ca-free sea water + 2.0 cc. alcohol

With the first three solutions, which served as controls, fertilization and normal development were 95–100 per cent. All of the lithium solutions were toxic to a certain degree. Fertilization and cleavage were approximately 75 per cent following treatment with solution 4, but only about 50 per cent with solution 8.

With solution 4, a few of the resulting larvae were normal (Figs. 1–2). Most of the forms, however, had shortened anal arms and inhibited oral lobes (Fig. 3). In some the anal arms were at widened angles (Fig. 4) and often askew (Fig. 5). Other larvae were poorly developed, with excessive mesenchyme and inhibited ventral regions (Figs. 6–9). There was an occasional exogastrula with partially recovered ectoderm (Figs. 10–12).

All the larvae from eggs treated with solution 5 developed inhibited oral lobes. In some the anal arms were small or absent, but in most they were fairly well developed although commonly unequal, askew, or at aberrant angles. There were a few extremely inhibited forms at the bottom of the culture that had developed but little past the gastrula stage and were filled with cells or cellular debris. From 10 to 15 per cent of the larvae were exogastrulae with definite entodermization and with ectoderms showing varying degrees of recovery (Figs. 10–12).

From 50 to 60 per cent of the eggs which cleaved after treatment with solution 6 developed into entodermized exogastrulae or entexogastrulae. Many of these showed considerable recovery of ectoderm (Figs. 10–12). The remaining 40–50 per cent of the eggs developed into cell-filled, inhibited gastrulae and inhibited plutei.

Treatment with solution 7 gave over 50 per cent radial, cell-filled exogastrulae with little or no differentiation of ectoderm (Figs. 13–14). The rest were cell-filled, inhibited gastrulae, with an occasional cell-filled, inhibited pluteus.

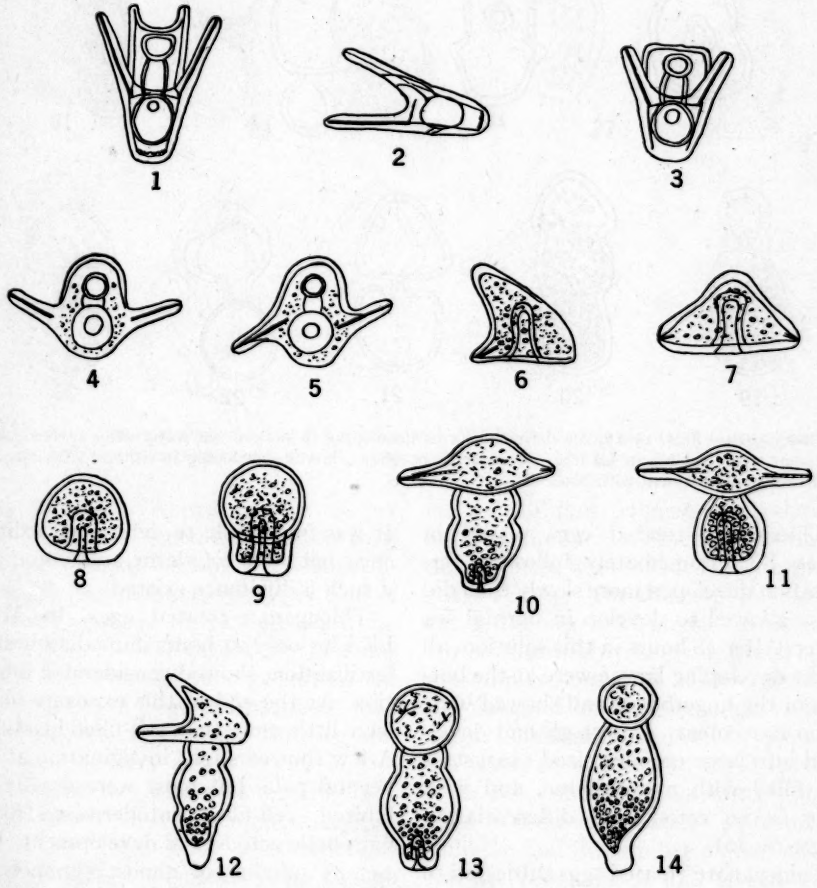
As mentioned above, approximately 50 per cent of the eggs failed to develop following exposure to solution 8. Of the remainder, over 75 per cent were cell-filled radial exogastrulae with no ectodermal differentiation (Figs. 13–14). The

[3] With slight modifications the Ca-free sea water was prepared according to Herbst's formula as follows: distilled water, 10,571 cc.; NaCl, 307 gm.; KCl, 8 gm.; $MgSO_4$, 66 gm.; $NaHCO_3$, 2 gm.

rest were inhibited gastrulae with an occasional inhibited pluteus.

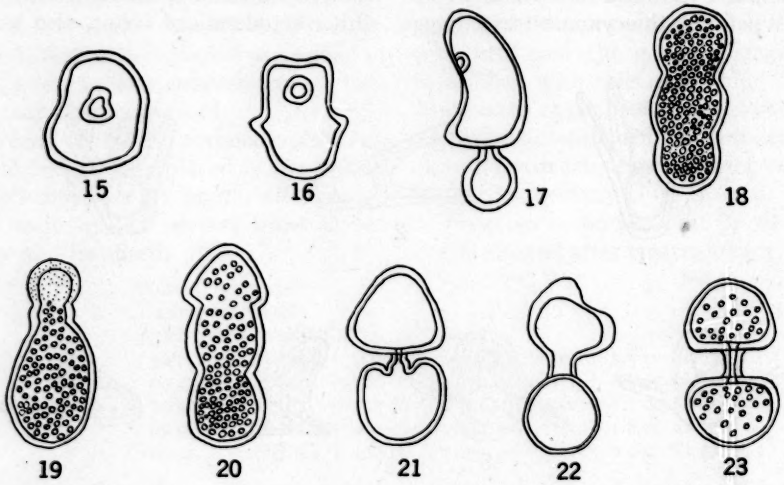
II. EXPERIMENTS INVOLVING A DOUBLE TREATMENT: SAND-DOLLAR EGGS EXPOSED TO THIOCYANATE BEFORE FERTILIZATION AND TO LITHIUM FOLLOWING FERTILIZATION

A large number of unfertilized eggs were placed in a solution of 20 cc. of 0.54 M NaCNS plus 100 cc. of Ca-free sea water for 12 hours. They were then washed and fertilized in normal sea water. When the thiocyanate-treated eggs were allowed to remain in normal sea water, they developed in a manner similar to that previously described (Rulon, 1941). After 48 hours, 30–40 per cent of the developing larvae showed ectodermization. Many of these ectodermized forms had developed a large stomodeum (Fig. 15) and contained neither mesenchyme nor gut. They were usually flattened ventrodorsally but otherwise showed no definite characteristic form. Other ectodermized forms, also without



FIGS. 1–14.—Figs. 1–2, normal plutei. Figs. 3–14, larvae developing in normal sea water after prefertilization treatment with increasing concentrations of lithium.

gut or mesenchyme, showed some ectodermal pattern (Fig. 16). A few had undergone exogastrulation, but no mesenchyme had appeared (Fig. 17). The remaining 60–70 per cent of the thiocyanate-treated eggs developed into larvae which ranged from grossly inhibited forms filled with mesenchyme (Figs. 6–8) up to normal plutei with well-developed oral lobes and anal arms.



FIGS. 15–23.—Figs. 15–17, ectodermized forms developing in normal sea water after prefertilization treatment with NaCNS in Ca-free sea water. Figs. 18–23, larvae developing in lithium solutions after prefertilization treatment with NaCNS.

Thiocyanate-treated eggs placed in M/40 LiCl immediately following fertilization developed more slowly than did those allowed to develop in normal sea water. After 48 hours in this solution, all of the developing larvae were at the bottom of the fingerbowls and showed little or no movement. Almost all had developed into large entodermized exogastrulae, filled with mesenchyme, and with little or no ectodermal differentiation (Figs. 18–20).

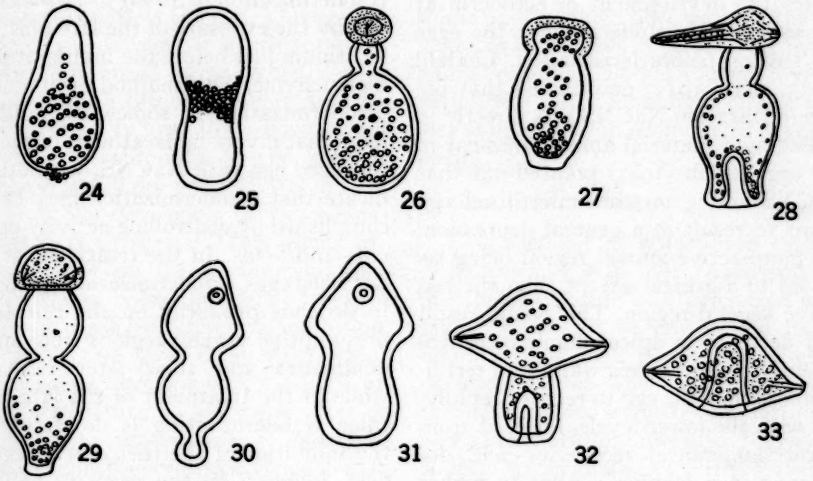
Thiocyanate-treated eggs subjected to M/50 LiCl after fertilization were less retarded in rate of development than were those in M/40 LiCl. After 48 hours most of the larvae were mesenchyme-filled exogastrulae, which showed some secondary invagination of the evaginated gut, as well as some differentiation of ectoderm. A small number of very curious forms appeared as a result of this treatment (Figs. 21–23). They were dumbbell shaped, were usually free of mesenchyme, and developed no pigment. It was impossible to determine a difference between ectoderm and entoderm, if such a difference existed.

Thiocyanate-treated eggs, in M/30 LiCl for only 24 hours immediately after fertilization, showed considerable inhibition. At the end of this exposure many were little more than cell-filled blastulae. A few showed slight invagination at the vegetal pole, but most were grossly inhibited, cell-filled entodermized forms with little ectodermal development (Fig. 24). A number of almost symmetrical forms with a band of mesenchyme near the center were noted (Fig. 25). When

these cultures were placed in normal sea water, almost all the larvae developed into extreme exogastrulae with tremendously extended entoderms and small apical knobs (Fig. 26). A few, however, were entirely free of pigment.

Thiocyanate-treated eggs, in M/40 LiCl for 24 hours after fertilization, had developed chiefly into elongated entodermized forms with pigmented apical ends (Fig. 27). Approximately 25 per cent showed no pigment, and ectodermal and entodermal regions were indistinguishable. Approximately 65 per cent of the entodermized forms had slight invaginations at their vegetal tips. Return to sea water at this time permitted the development of huge exogastrulae, with the ectodermal region often showing considerable recovery and with one or two anal arms heavy with skeleton (Fig. 28). Others showed less development of ectoderm (Fig. 29), while a considerable number lacked pigment even though skeleton was present.

Approximately two-thirds of the thiocyanate-treated eggs exposed to M/50 LiCl for 24 hours developed into exogastrulae with neither pigment nor mesenchyme (Figs. 30–31). The remaining larvae were inhibited forms with partial ectodermal inhibition (Figs. 32–33).



FIGS. 24–33.—Larvae treated before fertilization with NaCNS in Ca-free sea water and after fertilization with varying concentrations of lithium for 24 hours.

DISCUSSION

In the experiments involving the use of lithium before fertilization it is apparent that lithium, on penetrating the egg, not only produces a general inhibition of development but tends to level the gradient of activity. The animal pole no longer exerts the degree of physiological dominance it formerly possessed. Much of the presumptive ectoderm is lowered to the physiological level of presumptive entoderm. Fertilization and return to normal sea water do not lead to rapid recovery from the concentrations of this agent used in these experiments. It seems not unlikely that lithium has a great affinity for the cell colloids and is not easily thrown off when the eggs are returned to normal sea water and fertilized. Ca-free

sea water seems to be necessary in these experiments—possibly to permit a more rapid penetration of lithium through the cell membrane. Ethyl alcohol apparently lengthens the fertilizable life of the egg in Ca-free lithium solutions and thus permits a sufficiently long exposure period.

In these and previous experiments by the author and others it has been shown that NaCNS in Ca-free sea water promotes the development of ectoderm at the expense of entoderm when the eggs are treated before fertilization. Lindahl (1933, 1936, 1942) maintained that before fertilization NaCNS acts directly in extending a material animal gradient in the egg. Rulon (1941) pointed out that NaCNS acting on the unfertilized egg seems to result in a general depression, the more active animal region being retarded to a greater extent than the less active vegetal region. This would result in a decrease in apical dominance. Apparently, return to sea water and fertilization permit the egg to recover rapidly, but with the lower levels, released from apical dominance, recovering and developing in a manner similar to higher levels. The hypothesis that the ectodermizing action of NaCNS is in part a recovery phenomenon is indicated by the fact that thiocyanate used before fertilization ectodermizes but after fertilization it entodermizes, while lithium entodermizes both before and after fertilization. If both produce the same type of modification when used after fertilization but opposite types of modification when used before fertilization, the suggestion that recovery from thiocyanate is more rapid and more complete than recovery from lithium appears pertinent and logical, since recovery modifications are differential in the opposite direction from differential inhibitions. That loss of dominance is an important factor in ectodermization may be shown by the physical isolation of the vegetal half or less of early cleavage stages in sea-urchin eggs (Horstadius, 1935). Here considerable ectodermization results in consequence of absence of apical dominance. Ectodermization in recovery from thiocyanate may be a more extreme case with absence of dominance from the beginning of development.

Child (1936) produced considerable ectodermization of *Strongylocentrotus* larvae by the exposure of the late blastulae to lithium just before the immigration of mesenchyme. This method of producing ectodermization is somewhat different from that involving treatment of the unfertilized egg with NaCNS, yet both indicate that ectodermization may be accomplished by controlling activity gradients and fields. In the treatment of unfertilized eggs, ectodermization apparently depends primarily on the release of presumptive vegetal regions from apical dominance and their later recovery, while in the treatment of the late blastulae ectodermization is dependent on the inhibition of the then active vegetal field, followed by the recovery and development of ectoderm at the expense of entoderm.

In the present experiments it is evident that the entodermizing (lithium) treatment after fertilization does not always or entirely correct the ectodermizing (thiocyanate) treatment before fertilization. Instead, the double treatment gives many forms which show a combination of effects. All concentrations and exposure periods used in these experiments result in a high percentage of exogastrulae. When the exposure period to lithium is long (48 hours), there is usually a large surplus of mesenchyme. When the exposure period is shorter (24 hours) and the concentration of lithium high (M/30), cell-filled exogastrulae are still the rule. However, when the exposure period is

short (24 hours) and the concentration of lithium low (M/50), approximately two-thirds of the larvae are *mesenchyme-free* exogastrulae.

These data indicate that a long exposure period and a high concentration of lithium permit only a slow recovery in thiocyanate-treated eggs. Accordingly, apical dominance remains low, and the physiological levels of presumptive entoderm and mesenchyme are maintained throughout most of the egg. This results in the larvae developing chiefly into cell-filled entodermized forms. When the exposure time to lithium is short and the concentration is low, the recovery and activation of entoderm to the level of ectoderm is still not possible, but in two-thirds of the developing larvae mesenchyme fails to appear. This seems explicable only on the assumption that, while lithium holds the higher levels of the egg in check by differential inhibition, the less active level (that from which mesenchyme develops later) is little affected. On return to sea water after 24 hours, this mesenchyme level undergoes differential recovery and activation and, with decreased apical dominance, does not develop into mesenchyme at all but into cells characteristic of higher levels in the gradient system.

In the experiments involving thiocyanate before fertilization and only normal sea water after fertilization, a few mesenchyme-free exogastrulae (which possibly may be called "ectodermized" exogastrulae) appear (Fig. 17). Also in many of the experiments involving lithium after fertilization of thiocyanate-treated eggs, a number of mesenchyme-free forms develop. It seems not unlikely that these all involve recovery (or conditioning) and development of the presumptive mesenchyme region at a higher gradient level but do not necessarily involve the recovery and activation of entoderm to ectoderm. It may well be that this process is the first step toward the development of the bipolarity that Lindahl (1936) found in his treatment of sea-urchin eggs. Certainly, some cases indicate this (Figs. 21, 25), particularly in the early stages of development when mesenchyme apparently arises from a region midway between presumptive ectodermal and entodermal regions (Fig. 25). The writer has never found, however, a single case in which both poles of the developing larva have gone very far in the animal direction. Perhaps future investigation will throw more light on the subject of bipolarity in echinoderm development.

SUMMARY

1. Treatment of unfertilized eggs of *D. excentricus* with high concentrations of lithium chloride in a 1 per cent alcoholic Ca-free sea-water solution for 12 hours gives a high percentage of entodermized larvae when the eggs are returned to normal sea water and fertilized.

2. Treatment of unfertilized eggs with sodium thiocyanate in Ca-free sea water gives a high percentage of ectodermized larvae when the eggs are returned to normal sea water and fertilized.

3. Treatment of eggs with thiocyanate in Ca-free sea water before fertilization and with lithium after fertilization gives a combination of effects. If the exposure to lithium is severe, the eggs develop chiefly into mesenchyme-filled entodermized exogastrulae. If the exposure to lithium is light, most of the eggs develop into mesenchyme-free exogastrulae. The double treatment also gives certain forms which show indications of bipolarity.

4. Lithium used before fertilization appears to flatten the physiological gradient by differential inhibition of the apical region, with perhaps a general inhibition of the whole egg. On return to normal sea

water and fertilization, recovery is slow and incomplete, and more or less of the prospective ectoderm is inhibited to the level of entoderm and mesenchyme of early stages, that is, it is entodermized. With further progress of development and recovery, activation of the whole region now determined as entoderm and mesenchyme occurs with increase in size.

5. Thiocyanate also appears to flatten the gradient of the unfertilized egg with a loss of dominance of the apical region. In this case, however, a return to sea water and fertilization leads to rapid recovery, with all regions at the high physiological level of ectoderm.

6. High concentrations of lithium after fertilization retard recovery of thiocyanate-treated eggs. Weaker solutions of lithium, while inhibiting the more apical regions, apparently allow partial recovery of basal (mesenchymal) regions. This permits the region lowest in activity in the unfertilized egg to develop at a higher physiological level (i.e., of entoderm or ectoderm).

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RELATION BETWEEN BLACK AND RED MELANIN PIGMENTS IN FEATHERS

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MANY recent genetic and embryological investigations have been centered around differences in pigmentation because of the ease with which these differences may be followed. The color and pattern characteristics of the hair of mammals (see Wright, 1942) and of the feathers of birds (see Willier and Rawles, 1940 and 1944; and Lillie and Wang, 1943) have been particularly investigated. The genetic and morphological bases for differences in the color of the melanin pigments deposited have been carefully worked out in a number of cases; but, although there has been much speculation, practically nothing is known regarding the developmental and chemical relationships of these pigments. Until these processes are better understood, no satisfactory interpretation of the observed pigmentary changes can be offered.

Some information about these rela-

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tionships has been obtained through a study of pigments obtained from the feathers of several breeds of domestic fowl and pigments formed from various precursors *in vitro*. Although it was not possible to complete a series of experiments designed to provide more concrete information on this subject, certain conclusions may be drawn from the observations made and from scattered reports in the literature.

It is etymologically incorrect to speak of "red melanin" or "red melanophores," but it is convenient to group under the long-established term "melanin" several aklali-soluble pigments deposited in granular form by migratory cells. Our knowledge of the chemical composition of these pigments is extremely limited, and further information may show them to have quite varied chemical structures; but at the present time their similar solubilities, modes of production, and absorption spectra seem to justify such a grouping. Those commonly referred to as "red" are really various shades of tan or red-brown.

DEGREES OF OXIDATION OF BLACK AND RED MELANIN PIGMENTS

In explaining various pigment configurations, Humm (1942), Lillie and Wang (1943), and Wang (1943) have assumed that the red and brown melanins deposited in feathers represent intermediate products in the formation of black melanin. This idea appears to be based upon the experiments of Raper and his co-workers (see Raper, 1928 and 1932) on the *in vitro* oxidation of tyrosine.

It is true that a red substance, hallachrome (the quinone of 5, 6-dihydroxydihydroindole-α-carboxylic acid), is formed as an intermediary in the production of black melanin from tyrosine by tyrosinase. It can be readily detected only in acid solutions, as it is rapidly transformed into a colorless substance and then into black melanin in neutral or slightly alkaline solutions. This pigment, either alone or mixed with black melanin, is probably responsible for the various reddish and brown shades seen during the production of melanin from tyrosine in slightly acid solutions; but its properties are entirely different from those of the red melanin obtained from feathers and other natural sources. Hallachrome is unstable in alkaline solution and is destroyed by boiling, while red melanin is quite stable in alkaline solution and is resistant to boiling. Their solubilities are also quite different, the halachrome (see Evans and Raper, 1937) being readily soluble in several organic solvents in which the red melanin is relatively insoluble. In view of these important differences between hallachrome and red melanin, the work of Raper and his coworkers provides no basis for regarding red melanin as an intermediate in the formation of black.

The work of Arnow (1938) is more pertinent than that of Raper to a discussion of the relation between black and red melanin. After a long period of refluxing with 0.1N HCl, he obtained from red human hair an acid-soluble pigment which appeared to be identical with the pigment produced by oxidizing black dopamelanin with atmospheric oxygen in alkaline solution. The natural and synthetic red pigments were found to have the same solubilities and the same absorption spectra, in the range 460–700 mμ, in both acid and alkaline solutions. From these comparisons he concluded that the red pigment was an oxidation product of black melanin, as Görnitz (1923) had previously suggested.

A similar relation can be shown to ex-

ist between feather pigments. Most of the red pigment found in the feathers of domestic fowl is not acid soluble in its native state, but it can be easily extracted from the feathers by 0.01N KOH or pH 10.0 carbonate buffer without heating. Red pigment obtained in this way was compared with black pigment obtained by digesting feathers from the breast tract of Brown Leghorn capons or from Black Minorcas in 6N HCl. The melanin granules, which are not affected by this treatment, were centrifuged off, washed, and then dissolved in 0.1N KOH with the aid of heat.

Both of these procedures are superior to the frequently employed method of digesting the entire feather in alkali. A preliminary study showed that the melanin content of feathers varied from less than 1 per cent to a maximum of 15 per cent of the fat-free weight. Digestion with alkali therefore provides solutions in which 85–99 per cent of the organic material is made up of various decomposition products of keratin. These interfering substances were largely eliminated in the procedures outlined above.

The process of extracting the red pigment probably left it essentially unaltered, but the properties of the black melanin may well have been changed by the treatment required to get it into solution. In granular form the red pigment is much more soluble than the black, and on this basis Görnitz (1923) divided the melanins into "phaeomelanins," which are easily soluble in alkali, and "eumelanins," which are more difficultly soluble. After they have once been in solution, however, their solubilities at different pH's above 7.0 are very similar. This change in solubility may be attributed to the breakdown of some structural arrangement found in the granules, to an actual chemical change in the melanin, or to both. Unfortunately, it has never been found possible to study natural black melanins (with the exception of squid ink, which may have somewhat different properties) except after rather drastic treatment with alkali; consequently, data are not available for critically evaluating the effects of this treatment.

Transmittance curves for the various pigment solutions were obtained with a Coleman spectrophotometer, using a 30-mμ illumination slit. Matched square cuvettes were employed, and the extinction coefficients calculated from the relationship

$$E = \frac{1}{d} \log \frac{I_o}{I},$$

where I is the intensity of the light after passing through the solution and I_o is the intensity passing through the same thickness of pure solvent. For convenience in making quick comparisons, the concentrations of most of the solutions were adjusted to give approximately the same absorption at 380 mμ.

The absorption spectra of black and red melanins obtained by these methods from Brown Leghorn capon breast and saddle feathers, respectively, are shown in Figure 1 and are representative of the spectra of pigments obtained from several breeds. The curves for red and black differ primarily in the slope between 440 and 560 mμ. Bohren, Conrad, and Warren (1943) detected a similar difference in slope between the absorption spectra of black and red melanins, although it may be seen from Figure 1 that the range studied by them, 500–700 mμ, is not the most suitable for showing these differences.

With these two spectra as reference points, it was then possible to study the effects of oxidation upon the absorption spectra of these pigments. Hydrogen peroxide was used as an oxidizing agent,

and it was tested in various strengths on melanin granules and upon natural and dopamelanin in solution. Black melanin in solution was found to be quite sensitive to peroxide at room temperature, the degree of effect produced being dependent upon the concentration of peroxide used and the period of time allowed for the reaction (see Fig. 1). In all cases the changes produced were in the direction of red, i.e., an increase in the slope of the absorption curve. The resulting spectra did not seem to fall into any clearly defined groups; but all gradations, from absorption curves differing but little from the typical black form to those with a slope considerably greater than that of the natural red melanin, could be produced by varying the length and intensity of the oxidation. In all probability the final solutions were mixtures of varying amounts of several pigments of different degrees of oxidation. Similar changes were brought about much more slowly by merely allowing the melanin to stand in alkaline solution so that it could be oxidized by atmospheric oxygen. These absorption curves deviated from those of the natural red melanins in only one important respect. In the range 380–440 mμ the absorption curves for oxidized black melanin fall off more sharply than the curves for natural red (Fig. 1). This was found to be true irrespective of whether natural black melanin or dopamelanin was oxidized. The effect of oxidation on the absorption spectrum of red melanin was to increase the slope of the curves in the same way as for black. In no case was any tendency observed for red to be oxidized in the direction of black.

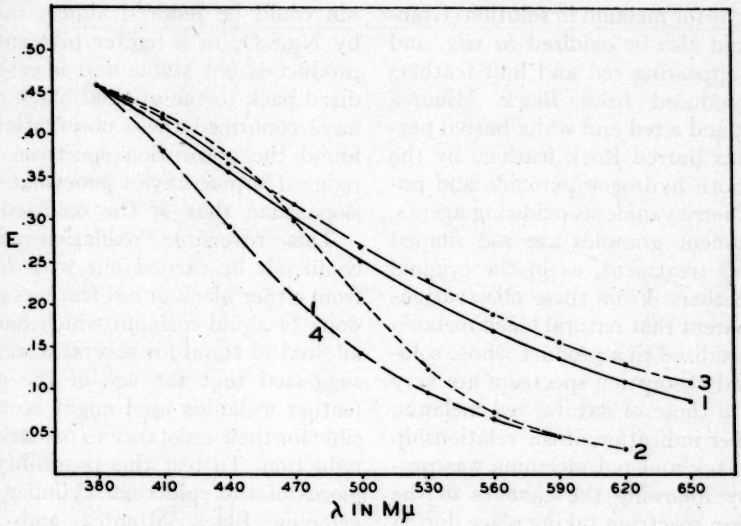

FIG. 1.—Extinction coefficients plotted against wave length for several melanins, all in 0.1 N KOH solution: *1*, natural black melanin from Brown Leghorn capon breast feathers; *2*, natural red melanin from Brown Leghorn capon saddle feathers; *3*, black melanin formed by autoxidation of dopa (the exact slope of the curve for such a pigment varies with the period of oxidation, the pH of the solution, etc.); *4*, the same pigment as No. 3 after standing with 1 per cent H_2O_2 at room temperature for three hours.

Black granules were found to be quite resistant to peroxide, but black melanine

in this form could also be oxidized. A suspension of black granules treated for 24 hours with 3 per cent H_2O_2 may be very quickly and easily dissolved in cold 0.01N KOH (similar to the reaction of natural red granules), although the absorption curve of the resulting solution is only slightly steeper than that of untreated black melanin. With longer oxidation, the curve is changed in the same manner as for melanin in solution. Granules could also be oxidized *in situ*, and typical-appearing red and buff feathers were produced from Black Minorca feathers and a red and white barred pattern from Barred Rock feathers by the use of both hydrogen peroxide and potassium ferricyanide as oxidizing agents. The pigment granules are rod shaped after this treatment, as in the original black feathers. From these observations it is apparent that natural black melanin can be oxidized to a product whose solubility and absorption spectrum are very similar to those of natural red melanin.

Another indication of the relationship of the black and red melanins was provided by following the changes in the absorption spectrum taking place during the production of black melanin from tyrosine and dopa. Both the oxidation of tyrosine by potato tyrosinase and the autoxidation of dopa were followed in these experiments. All experiments were run in phosphate buffers at pH 7.4. Samples were taken at various intervals after the beginning of the experiment and made up to 0.1N with KOH. The spectra were then obtained in the usual manner. A typical experiment of this type involving the autoxidation of dopa is shown in Figure 2. The slope of curve No. 4 is nearly the maximum for melanin formed in this manner and is similar to that of naturally occurring black melanin.

All the oxidative changes discussed above produce relatively stable products and seem to indicate that if the natural red and black melanins in feathers are formed from the same constituents, the red probably represents the more highly oxidized form. However, Figge (1939 and 1940*a*) focused attention upon a somewhat different aspect of this problem. He found that fresh dopa or squid melanin could be reduced almost instantly by $Na_2S_2O_4$ to a lighter pigment. This product is not stable and is easily oxidized back to the original black color. I have confirmed these observations and found the absorption spectrum of the reduced form to have a somewhat steeper slope than that of the oxidized form.

This reversible oxidation-reduction could not be carried out with melanin from either black or red feathers or with dopa or squid melanin which had been allowed to stand for several weeks. This suggested that the age of the natural feather melanins used might be responsible for their resistance to oxidation and reduction. To test this possibility, thin pieces of the epidermal cylinder of developing Black Minorca and Rhode Island Red feathers, taken from the base of the barb ridges where pigment was just beginning to be formed, were incubated for 12 hours in 3 per cent H_2O_2 or 3 per cent $Na_2S_2O_4$, with several changes of solution. The color of the pigment was carefully noted under a dissecting microscope both before and after treatment. The only color change observed was a partial oxidation of the black granules to a lighter color by the H_2O_2. In no case were red granules oxidized to black by the H_2O_2 or black granules reduced to red by the $Na_2S_2O_4$.

On the basis of the available evidence it is impossible to say definitely whether or not natural red melanin is more highly

oxidized than black melanin. There is no question but that lighter-colored products may be formed from fresh dopamelanin and some natural black melanins by both oxidation and reduction. Rothman (1942*a*) found a decrease in melanin concentration with continued ultraviolet irradiation of tyrosine solutions and concluded that this was "apparently an oxidation of melanin into lighter colored or colorless soluble products"; and in the other direction he observed (Rothman, 1942*b*) the reduction of the newly formed melanin to lighter-colored products by ascorbic acid in acid solution. Miescher and Minder (1939) also noted an increase in the intensity of the color of melanin pigment in human skin with oxidation and a decrease with reduction. However, the absence of transitional forms between red and black melanophores (see discussion, Nickerson, 1945), the spectral changes involved in the formation of melanin from tyrosine and dopa, and the fact that even newly formed red melanin in feathers cannot be oxidized to black make it appear improbable that red melanophores represent blacks in which the oxidative processes have been inhibited at an intermediate stage or that natural red melanin is an intermediate in the formation of black.

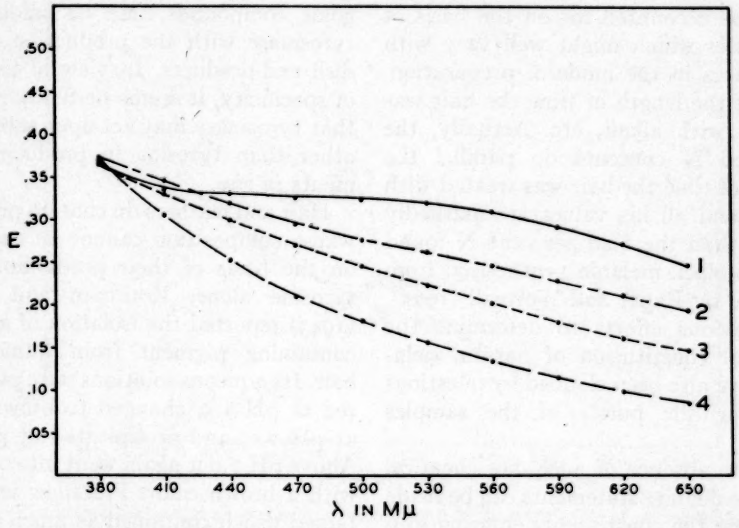

FIG. 2.—Extinction coefficients plotted against wave length to show the changes in absorption taking place during the production of melanin from dopa. Samples were taken at intervals from a solution of 0.5 mg/ml dopa autoxidized at 38° C. and pH 7.4, and the spectra determined in 0.1 N KOH. *1*, three hours' oxidation; *2*, six hours' oxidation; *3*, twenty-four hours' oxidation; and *4*, fifty-two hours' oxidation.

PIGMENTS PRODUCED FROM VARIOUS SUBSTRATES

It is evident that a number of difficulties arise in attempting to interpret the relation of black to red melanin on the basis that these pigments are formed from the same substrate and differ only in their degree of oxidation. There is, however, no really concrete evidence that these different, and probably related, pigments are produced from the same components. Neumann (1937) re-

ported that the yellow, brown, and black melanin from rabbit hair (increasingly difficult to get into alkaline solution in that order) contained 9.15, 11.77, and 12.36 per cent N, respectively. He concluded that these pigments represented a series formed from the same precursor. However, these results must be considered with caution as the melanin pigments are amorphous and difficult to purify and the observed differences could be accounted for on the basis of impurities which might well vary with differences in the mode of preparation, such as the length of time the hair was treated with alkali, etc. Actually, the observed N contents do parallel the length of time the hair was treated with alkali, and all his values are markedly higher than the 8.40 per cent N found in pure black melanin synthesized from tyrosine by Raper and Wormall (1925). All previous efforts to determine the chemical constitution of natural melanins have also been clouded by questions regarding the purity of the samples tested.

In the absence of adequate chemical data, no definite statements can be made regarding the constituents entering into the formation of different types of melanins. However, certain indications may be found in the results of less direct experiments and in a limited chemical knowledge of one component of red melanin.

Charles and Rawles (1940) demonstrated that both black (Black Minorca and hybrid black) and red (New Hampshire Red) feather germs contained tyrosinase and that the enzyme from both sources acted to produce black melanin from tyrosine *in vitro*. This is not surprising, as the only stable product definitely known to be formed by the action of tyrosinase on tyrosine is black. Tyrosinase, however, has only relative specificity. It has been known for many years that it will act on a great number of monohydric and polyhydric phenols other than tyrosine to yield various yellow, red, or reddish-brown pigments. Recently, Randall and Hitchings (1944) showed this to be true for many phenethylamine derivatives; and Graubard and Pincus (1942) demonstrated that the water-soluble esters of various estrogenic compounds may be oxidized by tyrosinase with the production of reddish end-products. In view of this lack of specificity, it seems perfectly possible that tyrosinase may act upon substances other than tyrosine in producing pigments *in vivo*.

Hair and feathers do contain pigments whose composition cannot be explained on the basis of their production from tyrosine alone. Rothman and Flesch (1943) reported the isolation of an iron-containing pigment from human red hair. Its aqueous solutions were purplish-red at pH 1.5, changed to brownish at at pH 2.6, and precipitated at pH 7.0. Above pH 7.0 it again went into solution with a brown color. Fractions were obtained which contained as much as 21.3 per cent iron. The authors concluded that this was a complex phenolic iron compound in which the phenolic–OH group was attached to a heterocyclic ring containing nitrogen. Unfortunately, they did not show the relation of this pigment to the red melanins in hair. However, this pigment is probably essentially the same as that from feathers described below and therefore could be shown to have many of the properties of the melanin group.

Previous to the appearance of their report, a similar pigment had been obtained from both red and buff feathers by extraction with 0.1N HCl at 45° C.

for several days. The absorption spectra of this pigment in acid (0.1N HCl) and alkaline (0.1N KOH) solutions are shown in Figure 3. The hump in the absorption curve for this pigment in acid solution agrees well with the absorption band at 535 mμ reported by Rothman and Flesch; and, in common with the absorption band of their solutions, this peak drops

component) could be detected in ash from the remaining red melanin, which was extracted with pH 10.0 carbonate buffer.

Although this pigment is distinct from black and the remainder of the red melanin, its absorption spectrum in alkaline solution is almost identical with that of the major portion of the red melanin,

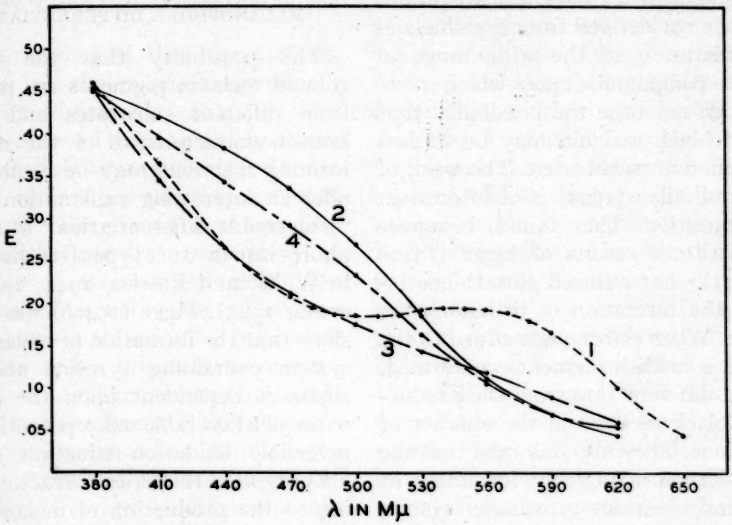

FIG. 3.—Extinction coefficients plotted against wave length to show the effect of acid and base on the absorption spectra of red melanins. *1*, Iron-containing pigment extracted with 0.1 N HCl from Buff Minorca feathers; spectrum determined in 0.1 N HCl solution. *2*, The same pigment as No. 1 in 0.1 N KOH solution. *3*, Iron-free red melanin extracted with pH 10.0 carbonate buffer (after removal of iron-containing fraction) and then treated with 0.1 N HCl for 2 hours at 100° C. to make it soluble in acid. Spectrum determined in 0.1 N HCl solution. *4*, The same pigment as No. 3 in 0.1 N KOH solution.

out in alkaline solution. As Rothman and Flesch found with their compound, this pigment is definitely more brownish in alkaline solution but is insoluble from pH 2.3 to pH 7.8. The apparent change in color at pH 2.3 is due to the first formation of a fine precipitate. All the color changes brought about by pH are reversible. Ash from this pigment gave a strong thiocyanate test for ferric ions, although only traces of iron (probably due to the presence of a little of this

and it is not unreasonable to assume that much of the molecule may be similar. After treatment with hot 0.1N HCl, most of the remaining iron-free red melanin can also be made soluble in acid. After this treatment its behavior is very similar to that of the iron-containing pigment (Fig. 3), and its acid and basic solutions even show a comparable spectral change in the region 380–500 mμ. The absence of the peak at 535 mμ in acid solution is the only marked difference. This acid-treated

red melanin is soluble in both acid and alkali and is insoluble between pH 2 and pH 5. The presence of iron-free red melanin altered in this manner may account for the difficulty experienced by Rothman and Flesch in purifying their samples and obtaining constant values for the iron content.

The demonstration of one melanin-like pigment in feathers which contains groupings not derived from tyrosine and a consideration of the wide range of phenolic compounds upon which tyrosinase can act raise the possibility that red and black melanin may be formed from different substrates. The work of Figge and Allen (1941) is of interest in this connection. They found, in agreement with the results of Figge (1940*b* and 1941), that reduced glutathione inhibited the formation of melanin from tyrosine. When estrone was added to the mixture, a reddish pigment was formed, but at a different rate from the production of black melanin in the absence of glutathione. They also observed that the rate and type of pigment formation in the tyrosine-estrone-tyrosinase system are, within certain limits, independent of the presence or absence of the glutathione. Unfortunately, the manner in which the concentrations of estrone and glutathione were varied by these authors makes it difficult to determine from their data the exact range of concentrations in which this is true. However, their observations seem to indicate that the type of pigment (reddish or black) produced was determined by the presence or absence of estrone. Although other interpretations are possible, it seems probable that the reddish pigment produced in these mixtures was formed, at least in part, from the estrone itself. I have found that the spectral curve of the reddish pigment produced by tyrosinase from a mixture of tyrosine and estrone is almost identical with that of the reddish pigment produced by tyrosinase from the estrone alone. Both are similar to, but have a somewhat steeper slope than, the spectral curve of black melanin produced from tyrosine.

RELATION OF MELANIN PRODUCTION TO MELANOPHORE DIFFERENTIATION

The possibility that the various-colored melanin pigments are produced from different substrates and certain known characteristics of the melanin-forming reactions may be combined to offer an interesting explanation for the "irreversible differentiation" of melanophores into distinct types (see discussions in Willier and Rawles, 1944; and Nickerson, 1945). Figge (1940*b*) was able to show that the formation of melanin in a system containing tyrosine and tyrosinase is dependent upon the maintenance of a favorable redox potential. Two reversible oxidation-reduction systems are present in the series of reactions leading to the production of melanin from tyrosine: dopa $\rightleftharpoons$ dopaquinone, $E_o = +0.372\ V$/pH 7 (Ball and Chen, 1933); and 5, 6-dihydroxydihydroindole-α-carboxylic acid $\rightleftharpoons$ hallachrome, $E_o = +0.022\ V$/pH 7 (Friedheim, 1933). The potential of the dopa $\rightleftharpoons$ dopaquinone system would almost completely inhibit the formation of melanin from tyrosine, but the dominant factor in maintaining the potential is the hallachrome system (see discussion by Figge, 1940*b*). In the absence of an artificial control of the potential of the reacting mixture by the addition of foreign substances, the potential maintained by this hallachrome system is very near to the optimum for the reaction. Once the formation of melanin from tyrosine has begun, these inter-

mediate products then act to hold the potential near the optimum for the continuation of the reaction. In other words, the system tends to stabilize itself. The mechanism of this stabilization is known for the system producing black melanin from tyrosine, and it is not unreasonable to assume that a similar process may be involved in maintaining a different set of conditions during the production of red melanin.

In the studies of the effects of sex hormones on the plumage of various breeds of fowl (see Nickerson, 1945), it has been observed that the melanophores fall into distinct types with regard to the pigment which they produce and that they do not change over to the production of another pigment, even when the conditions of their environment have been changed in such a way as to cause melanophores just beginning to produce pigment to form a different type of melanin. The maintenance of optimum conditions for a given pigmentation process by intermediate steps in the same process provides a possible explanation of this essentially "irreversible differentiation" of the melanophore precursors into red and black types. This interpretation would consider that the "differentiation" of these cells takes place at the same time as, and probably cannot be differentiated from, the beginning of pigment formation. The determination would be relatively irreversible because, once begun, the pigment-forming processes involve steps which maintain conditions near the optimum for their continuation.

Such a formulation is in agreement with the known facts, but there are obviously many factors which still require experimental confirmation. Estrone has been used as an example in much of the foregoing discussion because it has been shown to affect pigment production both *in vitro* and *in vivo*. Whether the sex hormones are actually built into the variously colored natural melanins or whether they act by modifying the conditions under which other materials are oxidized remains to be determined. In different breeds and feather tracts, the effects of sex hormones on the plumage color may be quite different, and this suggests that the effect produced is dependent upon the original condition of the reacting cells. This situation would be at least analogous to that reported by Figge (1940*b*) in Amphibia. He found that phenol indophenol could promote or inhibit melanin formation in different types of cells, depending upon the original redox potential of the cells.

Cell differentiation has been extensively studied and discussed in descriptive terms, but little or nothing is known of the actual mechanisms involved in causing a cell to develop in a particular direction or in preventing it from changing its course once a critical stage has been passed. The differentiation of melanophores into specific types appears to offer very favorable material for studying these problems, and the foregoing interpretation is an effort to explain this differentiation in terms of known characteristics of the processes involved in pigment formation. In the absence of critical data on several of the points discussed, this interpretation can be considered only tentative, but it does seem probable that the ultimate solution of this problem is to be found in the properties and characteristics of the pigmentation reactions themselves.

SUMMARY

1. The absorption spectra and solubilities of red and black melanins from feathers of several breeds of domestic fowl were investigated and the effects

of oxidizing and reducing agents upon these pigments were studied.

2. The oxidation of natural black melanin could be carried out so as to give it the same solubilities and essentially the same absorption spectrum as red melanin. Even by using newly formed natural melanins from the bases of barb ridges in developing feathers it was found impossible to oxidize the red to black or to reduce the black to red.

3. Changes in the absorption spectrum occurring during the oxidation of tyrosine and dopa were followed. The slope of the absorption curve was found to increase gradually until it was approximately the same as that for natural black melanin. This slope could then be increased to that characteristic of natural red melanin by the addition of H_2O_2.

4. Although lighter-colored reduction products may be formed from fresh dopa-melanin and certain natural melanins, it was concluded that naturally occurring red melanophores and melanins probably do not represent transitional stages in the formation of black.

5. An iron-containing component of red melanin obtained from red and buff feathers was described and shown to have characteristics very similar to those of the remainder of the red melanin which contains no iron. The production of variously colored "melanins" by tyrosinase from substrates other than tyrosine was discussed.

6. The possibility of explaining the irreversible differentiation of melanophores into distinct types in terms of known characteristics of the pigment-producing reactions was pointed out and discussed.

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CONDITIONS MODIFYING THE EXPRESSION OF SILVER IN THE SILVER CAMPINE FOWL[1]

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THE breeds and varieties of domestic fowl, excluding forms which are solid black or white, can be conveniently divided into two classes, those which normally have "red"[3] in their plumage and those which do not. This difference is due primarily to the action of a single sex-linked gene, silver (S), which inhibits the formation of red. Gold (s) has been generally assumed to be the allele of silver, but poultry geneticists do not agree on this point. There is some evidence from breeding experiments that the situation is complicated by the action of autosomal genes, but in the well-established breeds of poultry, the distinction between silver and nonsilver (gold) forms is generally quite clear.

Our knowledge of the genetic basis of these plumage differences is still incomplete, but even less is known regarding the mechanisms through which the known genes bring about their phenotypic effects and the conditions which may modify their expression. Some of the factors involved in the suppression of red by S in the Silver Campine will be considered here.

This breed is known to carry a dominant sex-linked gene for silver (Jull, 1940), a dominant autosomal gene for hen-feathering, and a recessive autosomal gene for barring (Punnett and Pease, 1921). The feathers of the normal adult are white and barred black and white (for a more complete description of this plumage see Nickerson, 1944). There is little sex dimorphism in the plumage. The Silver Campines used in the following experiments were from stock kept for one or two generations in the laboratory

[1] I wish to express my appreciation to Dr. B. H. Willier and Dr. Mary Rawles for helpful and stimulating discussion on this topic, and to Mr. John Spurbeck for the photographs used.

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[3] This designation includes the many variations of buff, gold, red-brown, salmon, etc., found in the plumage of the domestic fowl. All these colors are actually variations of tan or brown (such as found in the Rhode Island Red) and are due to melanin-like pigments. True reds are due to an entirely different group of pigments, which are not known to occur in the feathers of the domestic fowl.

and were good examples of this variety. All the birds were well barred, and no macroscopically visible red pigment was found in the adult plumage of over forty normal males and females raised from this stock. However, a microscopic examination of the developing feather parts showed them to contain a very few scattered red melanophores, and an occasional red-pigment granule was also found in the mature feather parts. The red melanophores were most frequently found in the primaries and secondaries.

In contrast to the adult plumage, the down plumage of this variety is irregularly mottled with red-brown. The development of this down plumage has not been studied, but the mature barbs contain large numbers of typical red-pigment granules (form shown in Fig. 16, and red melanophores are undoubtedly present during development.

EFFECTS OF CASTRATION

Seven Silver Campine males were caponized at about two months of age, and the subsequent plumage changes were observed. Feathers regenerating after castration differ from the normal Campine type in both shape and coloration. The shape of the Silver Campine capon feathers is similar to that of males or capons of normally cock-feathered breeds. The back, saddle, wing bow, and shoulder have the typical cock-feathered form with lacy edges. The tail coverts are increased in length, and typical sickle feathers are formed. The larger wing coverts, remiges, main tail feathers, and the feathers of the thigh region show the least alteration of the normal Campine shape, but they are somewhat longer.

The capon feathers contain less black than those of the normal Campine male, with the exception of the main tail feathers, in which the black is increased. The breast feathers are entirely white except for a single black bar near the tip and some diffuse black in the fluff regions (Fig. 3). The back and saddle feathers have no black pigment in the vane, except for small irregular patches along the rachis (Fig. 9), and the same reduction in the amount of black occurs to a variable extent in the feathers developing in the wing-bow (Fig. 1) and shoulder regions. The color of the large coverts, remiges, and the feathers of the thigh is least affected, and the capon feathers show only a moderate increase in the amount of white. A variable and often considerable amount of black pigment is retained in the fluff of all feathers.

The general features of this response to castration have been described and figured by Morgan (1920), Emmens and Parkes (1940), and others; but one important feature not mentioned by previous authors is the appearance of red pigment in the developing feathers. This was noted in all the castrated birds, although the amount of red present varied considerably in different individuals. The red pigment is most apparent in the feathers of the tail covert, saddle, back, shoulder, and wing bow, and its appearance is more than a mere unmasking of pigment from the occasional red melanophores present in the normal adult feathers. In normal birds very few red melanophores are present, and these are most frequently found in the developing primaries and secondaries, while these feathers, although they have increased amounts of white in the capon, seldom show macroscopically visible red pigment. In most of the capon feathers the red pigment is widely scattered, giving the white areas a yellowish or reddish cast, but patches of red pigment as dark as that found in the typical Rhode Island or New Hampshire Red plumage are frequently found. These dark-red patches are particularly apparent in the tail

coverts (see Fig. 12), where there is little decrease in the amount of black and the pigment is too dense to have been completely masked by black in the normal male plumage.

A microscopic examination of split preparations and sections of regenerating capon feathers showed that this red pigment is deposited by large numbers of typical red melanophores (Fig. 15). These contain small, round, light-colored granules (Fig. 16) and are easily distinguished from the black melanophores, which in the Campine usually contain quite long black granules (Figs. 13 and 14).

EFFECTS OF SEX HORMONE INJECTIONS

The injection of adequate doses of either male or female sex hormone into Silver Campine capons leads to a return of the normal shape and pigmentation of the regenerating feathers, including the absence of all macroscopically visible red pigment. Feathers were plucked from various regions of four capons a little over one year old, and the birds were then injected subcutaneously every 2 days with the following doses of hormone in oil: (1) 0.24 mg. estradiol monobenzoate, (2) 0.96 mg. estradiol monobenzoate, (3) 0.2 mg. testosterone propionate, and (4) 1.0 mg. testosterone propionate. These injections were continued for a period of 16 days (8 injections) in all the series except No. 2, which was continued for 30 days (15 injections). Regenerating feathers were plucked at 22, 42, and 48 hours and 3, 4, 5, and 9 days after the first injections. These feather germs were split, fixed, dehydrated, and mounted for a study of the changes taking place in the arrangement and color of the melanophores. Other feathers were allowed to complete their regeneration and were then plucked and studied.

All the series of injections except No. 3 caused a very marked change in the pigmentation of the developing feathers. The feathers of bird No. 2 regenerating during the period of hormone injections became almost indistinguishable from those of a normal adult Campine (cf. Figs. 7 and 8), and the regenerating feathers of Nos. 1 and 4 developed the normal coloration and pattern during the period of the injections. These latter injections did not cover the entire period of growth of the individual feathers, which, therefore, are mosaics of the capon and normal male coloration and form (Figs. 2, 4, and 6).

A microscopic examination of split preparations of feather germs from the saddle and wing bow showed that a few black melanophores were present in preparations made 22 hours after the first injection. Scattered melanophores were found in the collar and a few in the bases of the barb ridges at this time. All or almost all of these contained small spherical black granules rather than the more typical rods. The black pigment usually appeared first on the ventral side of the feather and then gradually spread toward the rachis, but this was not always the case. In later stages (Figs. 10 and 11) melanophores in the regions which had first become pigmented were found to contain rod-shaped granules, while those in regions in which pigmentation had just begun contained spheres. This would seem to indicate that round granules were the first to be formed when conditions became appropriate for the production of black pigment. After 9 days the feathers from Nos. 1, 2, and 4 had formed at least one complete bar, and the pigmentation picture was essentially the same as in the normal adult plumage.

It is of interest that both androgens and estrogens are effective in modifying the feather pigmentation in Silver Cam-

pines. Although the estrogens have been shown to have a marked effect on the melanophores of several breeds and varieties of domestic fowl, the androgens have been reported to have little effect on the coloration. The different situation in the Silver Campine may depend partially upon the change in feather form which the male hormone effects in hen-feathered breeds, but radical changes in pigmentation also occur in the breast tract, where the feather form is not markedly altered.

The primary effect of the gene silver in poultry is to inhibit the development of red melanophores and the deposition of red pigment in the feathers. In the normal adult Silver Campine this suppression of red is nearly complete. A very few pale-red melanophores are present in the developing feathers, but they deposit little or no pigment in the feather parts. The foregoing observations indicate that this action of S is much less complete in the absence of an adequate sex hormone level in the body; but the effect of S is not completely abolished in the absence of sex hormones, as the feathers of Silver Campine capons still have much less red pigment than Golden Campine feathers. The sex hormones themselves have not been shown to suppress red-pigment deposition in the domestic fowl, and consequently the effect reported here cannot be considered as an additive effect. It is rather a potentiation of the red-suppressing action of the gene S by a factor which in itself does not suppress red.

RELATION OF AGE TO THE EFFECT OF SEX HORMONES ON THE EXPRESSION OF SILVER

The observed relationship between the presence of sex hormones and the suppression of red in the adult Silver Campine suggested that a very low hormone concentration during embryonic development might account for the large amounts of red pigment found in the down plumage. To test this assumption, various doses of male hormone (testosterone propionate) in oil were injected into the albumin of 14 Silver Campine eggs between the fourth and eleventh days of incubation. Almost the entire growth of the down feather takes place between the eleventh and seventeenth days of incubation (Watterson, 1942), and the injections were arranged to allow for hormone action during this period. Data regarding those chicks which developed far enough to show the presence or absence of red in the down plumage are given in Table 1.

TABLE 1

CHICKS TREATED WITH TESTOSTERONE PROPIONATE DURING EMBRYONIC DEVELOPMENT

Chick Number	Age Injected (Days)	Dose (Mg.)	Time Observed
1	11	0.3	Hatched
2	10	0.3	Hatched
3	10	0.5	Hatched
6	11	0.5	Hatched
7	11	0.8	Died at hatching (20 days)
8	10	0.5	Hatched
9	7	0.5	17 days
11	4	0.5	19 days
13	5	0.3	19 days

It has been reported (van Oordt and Rinkel, 1940) that a dose of 1.5 mg. dehydroandosterone administered during embryonic development will produce marked pathological changes in the mesonephros and Wolffian ducts; and Dantschakoff (1938) observed similar effects with 0.5 mg. of testosterone propionate. In this experiment it was desirable to have the chicks hatch, and consequently the dosages were kept below the level which might be expected to cause marked urinogenital abnormalities. However, a dose of 0.8 mg/egg is, on a weight basis,

roughly forty times as great as a single injection given to capon No. 4, and five times as great as the total injections of this series. The plumage of capon No. 4 developing during the period of the injections contained no visible red, and this change from the capon type of pigmentation was probably complete within 48 hours, i.e., under the influence of the hormone concentration produced by a single injection. In all cases the combs of the newly hatched chicks were markedly enlarged and turgid as a result of the hormone action.

Red pigment was present in all the chicks which developed far enough for the color of the down plumage to be determined, and the treated chicks which hatched had a down plumage indistinguishable from that of normal Silver Campine chicks. It is possible that still larger doses of hormone might have brought about an inhibition of red pigment; but it is probably safe to assume that the ability of the male hormone to suppress red in the Silver Campine is conditioned by the age of the tissue upon which it acts. It was previously shown (Nickerson, 1944) that melanophores obtained from $3\frac{1}{2}$–4-day-old Silver Campine embryos and from developing barb ridges of adult feathers grafted into White Leghorn embryos produced essentially the same coloration in the down plumage, including red in both cases. In the light of this fact it seems probable that it is the age of the epidermal components of the developing feather rather than of the melanophores which is of importance in regulating the appearance of red in the plumage of Silver Campines. In this case, therefore, the hormones may be considered to be acting upon the melanophores through the epidermal substratum, or at least to have their action on the melanophores conditioned by this substratum.

RELATION BETWEEN BLACK AND RED MELANOPHORES

The presence of two or more distinct types of melanophores in the plumage of the Silver Campine and many other breeds of domestic fowl and the marked changes in their relative numbers and distribution which occur with age and hormonal changes have given rise to much speculation regarding the developmental relationships of these types. Willier (1942) and Willier and Rawles (1944) have suggested that the two types may differentiate directly from a common stem cell and that this differentiation is then irreversible. This is in contrast to the view, expressed particularly by Humm (1942), Lillie and Wang (1943), and Wang (1943), that red melanophores are merely blacks whose development has been arrested before they reached the black stage.

There is really little concrete evidence to substantiate this second interpretation. Humm (1942) did report that pure cultures containing nothing but "fully differentiated" reddish-brown melanophores from a Rhode Island Red donor produced both black and red pigment when grafted to White Leghorn hosts. If this observation is correct, it indicates that even after the beginning of pigment formation the type of pigment produced can be altered and the red melanophores changed to black. However, no evidence other than the gross appearance of the dense masses of melanophores was presented to show that these cultures did not also contain undifferentiated pigment cells. That such undifferentiated cells were present and largely responsible for the pigment produced is indicated by the fact that the ability of these cultures to pigment the host feathers decreased gradually to zero as the cultures aged, although some living differentiated

cells were presumably present in all the cultures used for grafting.

The distinctness of the melanophore types is indicated by the histological studies of Danforth (1937) and Hamilton (1941), as well as those reported here. Although several types of melanophores were observed in these studies, each contained only a single type of pigment granule, and no evidence for the conversion of melanophores from one type to another was found. This indicates that, after pigmentation has begun, the black and red melanophores are distinct, but it provides no evidence regarding the stage in development at which these differences arise. Hamilton considered that the segregation into black and red melanophore precursors occurred quite early in embryonic development; but this interpretation meets a number of difficulties, particularly (as pointed out by Danforth, 1937) in the promptness and completeness of the color changes elicited by hormones. In Brown Leghorns (Lillie and Juhn, 1932), as well as in Silver Campines, melanophores producing one kind of pigment may be completely replaced by those producing another within less than 24 hours after the injection of hormone in oil. Both before and after such a change the barb ridges may be densely packed with pigment cells. It therefore seems improbable that the hormones are merely acting selectively upon two types of melanophores previously determined with respect to type. Willier and Rawles (1944) have considered that the melanophore precursors are fixed as either black or red after entering the epidermis of the developing feather. The time of determination is not more specifically localized, but the reference to "precursor melanophores" indicates that they believed it to occur at some time before the beginning of pigment formation.

There is at present no critical evidence regarding either the exact time or the mechanism involved in this irreversible determination of melanophore types. However, known characteristics of the pigment-producing reactions (see discussion, Nickerson, 1945) offer some indirect evidence that the beginning of pigment production may itself be the critical event in fixing the melanophore type as black or red.

SUMMARY

Regenerating and mature Silver Campine feathers from normal males and females, capons, and capons after the injection of male and female hormones were studied. Red pigment, not present in the normal plumage, appeared in feathers developing after castration, but its production was suppressed by the injection of either male or female sex hormones. These changes in pigmentation were correlated with the numbers of black and red melanophores present in the developing feather germs. The complete expression of silver (S), suppression of red, in this variety appears to be dependent upon an adequate sex hormone level.

The injection of testosterone propionate into the albumin of Silver Campine eggs between the fourth and eleventh days of incubation failed to inhibit the appearance of red pigment in the down plumage. This indicates that an age factor is also involved in the expression of silver.

Possible developmental relations of black and red melanophores are discussed in the light of the changes produced by hormone injections.

PLATE I

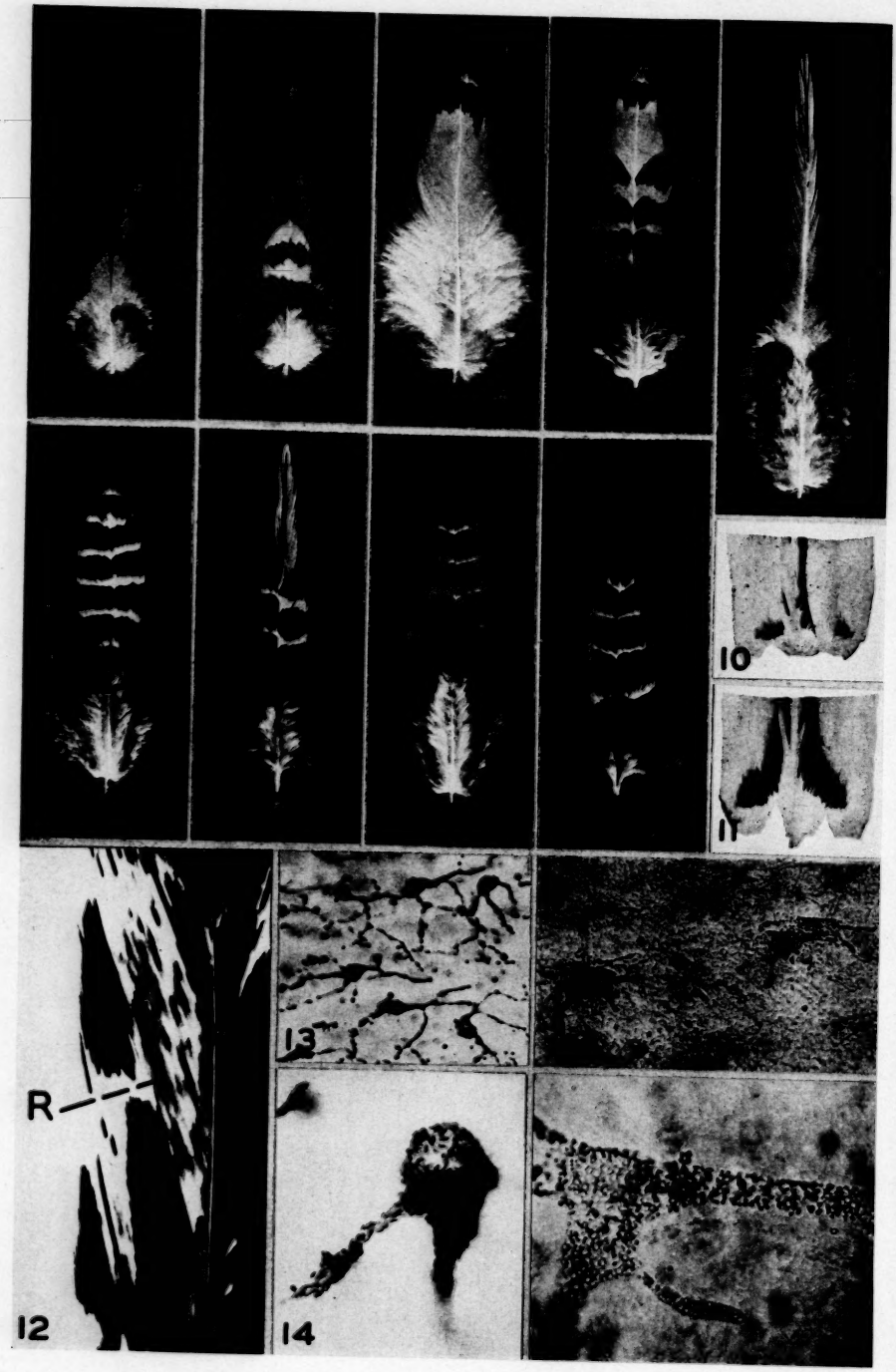

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PLATE I

(All from Silver Campines)

FIG. 1.—Feather from wing bow of capon No. 1, showing normal capon type. ×½.

FIG. 2.—Feather from wing bow of capon No. 1. Basal one-half of feather was grown during the period of hormone injection. ×½.

FIG. 3.—Breast feather from capon No. 4, showing normal capon type. ×½.

FIG. 4.—Breast feather from capon No. 4. Basal two-thirds was grown during the period of hormone injection. ×½.

FIG. 5.—Breast feather from normal male. ×½.

FIG. 6.—Saddle feather from capon No. 2. Basal one-half was grown during the period of hormone injection. ×½.

FIG. 7.—Saddle feather from normal male. ×½.

FIG. 8.—Saddle feather from capon No. 2. Entire feather was grown during the period of hormone injections. This feather was plucked before the fluff was completely formed. ×½.

FIG. 9.—Saddle feather from capon No. 2, showing normal capon type. ×½.

FIG. 10.—Basal part of a split preparation of a feather germ from the wing bow of capon No. 2, plucked 42 hours after the beginning of hormone injection. ×3.

FIG. 11.—Basal part of a split preparation of a feather germ from the wing bow of capon No. 2, plucked 72 hours after the beginning of hormone injection. ×3.

FIG. 12.—Part of the vane of a capon tail covert, showing areas of dense red pigment (*R*). ×1.

FIG. 13.—Black melanophores from the base of a regenerating capon saddle feather. ×210.

FIG. 14.—Black melanophore from the base of a regenerating capon saddle feather. Note rod-shaped granules. ×970.

FIG. 15.—Red melanophores from the base of a regenerating capon saddle feather. ×210.

FIG. 16.—Red melanophore from the base of a regenerating capon saddle feather. Note the small round granules. ×970.

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

AGING AND DEGENERATIVE DISEASES,[1] EDITED BY ROBERT A. MOORE, ASSISTED BY WILLARD M. ALLEN, E. V. COWDRY, WILLIAM B. KOUNTZ, AND W. BARRY, JR.

Reviewed by A. J. CARLSON
University of Chicago

THIS book contains significant contributions by thirteen investigators. Leo Loeb outlines our present knowledge of the relation of aging to cancer. John A. Saxton, Jr., discusses the relation of food and nutrition to the life-span of the rat. William de B. MacNider develops the importance of increased understanding of the causes and processes of aging both to science and to society. But the central theme of the volume is the causes and consequences of the aging processes in the cardiovascular system, that is, sclerosis of the blood vessels (chaps. i–x). Competent investigators deal with different aspects of this great unknown, such as lipid metabolism (Irvine H. Page), morphology (W. C. Hueper), pancreas hormones (L. R. Dragstedt), blood cholesterol (Kountz, Sonnenberg, Hofstatter, and Wolf). Dr. Edward J. Stieglitz outlines present difficulties in clinical diagnosis of the degenerative diseases of aging, and Dr. William J. Kerr points out our present unsatisfactory therapy of these maladies. In brief, the problem is here. It is increasing in importance, socially and economically. The answer is more and better research on the nature of the aging processes. We must know more before we can do better, either in delaying or in treating the degenerative diseases of aging. This volume is of equal interest to students of heredity, pathology, biochemistry, physiology, and medicine, since, in the nature of things, the biological problems of aging, in the individual and in the species, must, for some time to come, be elucidated by experiments on animals.

TEMPO AND MODE IN EVOLUTION,[2] BY GEORGE GAYLORD SIMPSON

Reviewed by HERLUF H. STRANDSKOV
University of Chicago

THE last few years have witnessed a veritable flood of books and papers on the subject of evolution. The most recent of the books is *Tempo and Mode in Evolution*, by George Gaylord Simpson, of the American Museum of Natural History. This book is a particularly welcome addition to the series on evolution because it presents the facts and opinions of a modern paleontologist. Most of the other books have been written by geneticists.

Simpson early in his book confesses to inadequate knowledge of genetics.

[1] Lancaster, Pa.: Jacques Cattell Press, 1945. Pp. 242. $3.00.

[2] New York: Columbia University Press, 1944. Pp. xviii+237. $3.50.

That he is not primarily a geneticist is obvious throughout the discussion, but, as he points out in the Introduction, many geneticists have written on the subject of evolution who lacked to an equal degree a knowledge of paleontology. In science we must expect such inadequacies and applaud anyone who has the courage to present his side of the picture even though he is not an authority on all aspects of the total field. Only by tolerance and co-operation of this type can the broad principles of science be developed.

It is not fitting in a brief review such as this to deal critically with the issues which are discussed in the book which is reviewed. I shall, however, comment on one or two points as I outline the contents of the chapters.

Chapter i deals with rates of evolution. This chapter is particularly rich in good illustrations from paleontology. A laudable feature is that these illustrations are presented in statistical form. The general conclusion is that no single law of rates can be formulated.

The second chapter deals with the determinants of evolution and includes a discussion of variability, mutation rate, and character of mutation. One notable feature of this chapter is that Simpson finds little or no support among fossil records for Goldschmidt's contention that evolution is primarily one of macroevolution. This chapter, however, also presents an opinion with which the present writer is in disagreement. Simpson properly separates variation of individuals within a group and variation between groups, but he contends that the roles of selection on the two are entirely different. He holds that "the action of natural selection on intragroup (or inter-individual) variation is essentially an originating force: it produces definitely new sorts of groups (populations)," whereas "action on intra-individual variation—can only eliminate, not originate, types of individuals or of individual reactions." In the opinion of the writer the action in each instance is one of originating new groups. Space does not permit a detailed analysis.

Chapters iii–v discuss "Types of Evolution," "Low-Rate and High-Rate Lines," and "Inertia, Trend, and Momentum." These chapters are excellent, especially in that they marshal paleontological data in support of points of view. Chapter vi is headed "Organism and Environment." In this chapter the author classifies the environment and discusses such questions as pre-adaptation and post-adaptation. The final chapter concerns "Modes of Evolution." The author distinguishes three modes: (1) speciation, (2) phyletic evolution, and (3) quantum evolution. He recognizes properly that these modes overlap but finds them suitable for discussion purposes. The term "quantum evolution" probably is new to most evolutionists, but after reading the type of evolution that it includes, one is inclined to agree that it is an appropriate term despite its usage in physics in a somewhat different sense.

All in all, the book is stimulating and rich in paleontological illustrations and should not be missed by anyone who is seriously interested in the subject of evolution.

MANOMETRIC TECHNIQUES AND RELATED METHODS FOR THE STUDY OF TISSUE METABOLISM,[1] BY W. W. UMBREIT, R. R. BURRIS, AND J. F. STAUFFER

Reviewed by SEVERO OCHOA
New York University College of Medicine

IN ADDITION to general manometric and chemical techniques, this book includes descriptions of special methods, manometric as well as analytical and preparative, contributed, besides the authors, by P. P. Cohen, G. A. Le Page, V. R. Potter, J. W. Bain, H. F. Deutsch, A. N. Hanson, H. A. Lardy, A. L. Lehninger, and W. C. Schneider—all of the University of Wisconsin.

The first six chapters, devoted to the Warburg constant-volume respirometer, cover both its theory and its practical applications in a very comprehensive manner, while the differential manometer is dealt with in chapter vii, with special consideration of its use in studies on photosynthesis. Some special manometric techniques, including the methods of Dickens and Simer, Dixon and Keilin, and the Summerson manometers, are described in chapter xi.

Chapter viii deals with methods of preparing for study animal, plant, and bacterial tissues, such as slices, minces, extracts, and dry powders; while the now extensively used homogenates of animal tissues are described in chapter ix. This chapter also includes methods of assay of some respiratory enzymes, such as succinic dehydrogenase, cytochrome oxidase, malic dehydrogenase, etc. Also a method for the assay of adenosinetriphosphatase and an outline of a method of study of oxidative phosphorylation in tissue homogenates are included. The use of creatine as the phosphate acceptor recommended for this type of study is, in the reviewer's opinion, less satisfactory than that of glucose, both because estimation of phosphate esterification is more rapid with the latter and because the formation of hexose diphosphate, contrary to that of phosphocreatine, is not reversible. In chapter x are described colorimetric methods for the estimation of nitrogen, sugar, and lactic, pyruvic, and citric acids. The titrimetric methods for sugar developed by Shaffer, Hartman, and Somogyi might well be used with advantage in some cases, and it is felt that a description of such methods would have been a welcome addition to the chapter. Regarding the estimation of pyruvic acid, the improvements and simplifications introduced by Friedemann and Haugen (Jour. Biol. Chem., 147:415, 1943) would seem to make this method more advantageous than the one described. Chapter xii describes the Thunberg methylene blue technique of studying the action of dehydrogenases and a simple, in many cases preferable, photometric estimation of methylene blue reduction. Chapter xiii deals with the estimation of dissolved oxygen, using the dropping mercury electrode, and with the potentiometric determination of oxidation-reduction potentials.

An excellent chapter on manometric estimation of metabolites and enzyme systems (chap. xiv) would have gained by completing it with a description of the methods developed by the Warburg school for determination of the reduction of pyridine nucleotides with activated hydrogen and with hydrosulphite, oxidation with ferricyanide, microestimation

[1] Minneapolis, Minn.: Burgess Pub. Co., 1945. Pp. 198. $3.50.

of amino nitrogen, and the ferricyanide method of studying the activity of some dehydrogenases. The determination of pyridine nucleotides has merely been sketched; a more complete description of manometric methods of determining diphosphopyridine nucleotide would have been welcomed by many workers. In chapter xv is described in detail a method of fractionation and analysis of phosphorylated intermediates of tissue metabolism. The reviewer finds that the method described for total phosphorus requires an extremely long time of ashing and that estimation of phosphate by the Fiske and Subbarow method in samples that have been previously treated with acid, alkali, iodide, etc., may not be very satisfactory, owing to the sensitivity of the method to salt. In such cases the method of Lohmann and Jendrassik (Biochem. Zeitschr., 178:418, 1926) would seem much more convenient. Chapter xvi, the last of the book, deals with the preparation of physiologically important intermediates and metabolites. Regarding the preparation of hexose phosphates, the synthetic methods of Levene for fructose-1-phosphate and glucose-6-phosphate are, in the reviewer's experience, simpler and give better yields than methods of preparation from biological material. Also the method described some years ago by R. A. Peters for preparing crystalline sodium pyruvate, using redistilled pyruvic acid and acetone, is thought to be superior to the one described in this chapter. As to the preparation of cytochrome c, the reviewer agrees with the statement that the obtaining of this in solid form by precipitation with acetone is unsatisfactory, but lyophylization gives excellent results.

The description of the various techniques is extremely clear and the inclusion of many illustrations, specific examples, and, occasionally, of actual experimental data will be found very helpful.

The book that is primarily intended to serve as a manual for graduate students and beginners in the field of tissue metabolism will also be useful to the specialist because of the wide field covered and the comprehensive references to the original literature.

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